(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 November 2002 (28.11.2002)

PCT

(10) International Publication Number
WO 02/095002 A2

(51) International Patent Classification[7]: C12N

(21) International Application Number: PCT/US02/16295

(22) International Filing Date: 22 May 2002 (22.05.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/292,845 22 May 2001 (22.05.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/292,845 (CON)
Filed on 22 May 2001 (22.05.2001)

(71) Applicant (for all designated States except US): UNIVERSITY OF CHICAGO [US/US]; 5640 South Ellis Avenue, Chicago, IL 60637 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): KAZMIERCZAK, Krystyna, M. [US/US]; 640 Forestview Avenue, Park Ridge, IL 60068 (US). DAVYDOVA, Elena, K. [RU/US]; 5623 S. Cottage Grove, #1, Chicago, IL 60637 (US). ROTHMAN-DENES, Lucia, B. [US/US]; 5830 South Stony Island Avenue, Apt. 9A, Chicago, IL 63637 (US).

(74) Agent: HANSON, Robert, E.; Fulbright & Jaworski L.L.P., 600 Congress Avenue, Suite 2400, Austin, TX 78701 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 02/095002 A2

**(54) Title:** N4 VIRION SINGLE-STRANDED DNA DEPENDENT RNA POLYMERASE

**(57) Abstract:** A histidine-tagged, deletion mutant of bacteriophage N4-coded, virion RNA polymerase (mini-vRNAP) which is active has been developed. The his-tagged mini-vRNAP has been cloned under the control of the Pbad promoter, is stable and is purified in a single step yielding large amounts (10 mg/liter of *E. coli* expressing cells). This RNA polymerase uses single-stranded DNA containing 17 bases (the promoter) upstream of the transcribed regions as a template. In the presence of *E. coli* SSB protein, it transcribes this template efficiently, providing a unique system to synthesize RNAs of the desired sequence using single-stranded DNA templates. The enzyme incorporates derivatized nucleoside triphosphates with high efficiency. A mutant of mini-vRNAP has been generated that incorporates deoxynucleoside triphosphates. In addition, the inventors have developed an *in vivo* system to express RNAs and proteins under mini vRNA polymerase promoter control.

# N4 VIRION SINGLE-STRANDED DNA DEPENDENT RNA POLYMERASE

## BACKGROUND OF THE INVENTION

This application claims the priority of U.S. Provisional Patent Application Serial No. 60/292,845, filed May 22, 2001, the entire disclosure of which is specifically incorporated herein by reference. The government may own rights in the present invention pursuant to grant number R01 A1 12575 from the National Institute of Health.

### I. Field of the Invention

The present invention relates generally to an RNA polymerase. More particularly, it provides a bacteriophage N4 virion RNA polymerase for synthesis of RNAs of desired sequences using single-stranded DNA templates.

### II. Description of Related Art

The expression of a protein-encoding gene in a host cell involves transcription of messenger RNA (mRNA) from DNA by an RNA polymerase enzyme. Subsequently the mRNA is processed, involving recognition of a region of the 3' UTR and addition of a tail of polyadenylate nucleotides to the 3' end of the mRNA by polyadenylation enzymes. After transcription, the mRNA encounters ribosomes which associate with a region of the 5' UTR of the mRNA and translocate in a 3'-ward direction along the mRNA. During translocation, amino acids are added to one another in sequence to form the polypeptide product of the protein-encoding gene. For prokaryotic transcription-translation, the Shine-Dalgarno sequence of the bacterial mRNA located about six to nine nucleotides before the initiation site for translation may be used for ribosome loading. This sequence is complementary to a sequence on the 3' end of the 16S rRNA and stimulates ribosome binding to the mRNA. The base pairing between the Shine-Dalgarno sequence and the mRNA sequences serves to align the initiating AUG for decoding.

Transcription of DNA into mRNA is regulated by the promoter region of the DNA. The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA, and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding complementary strand of RNA. RNA polymerases from different species typically recognize promoter regions comprised of different sequences. In order to express a protein-encoding gene in a host cell, either the promoter driving transcription of the protein-encoding gene must be recognized by a host RNA polymerase, or an RNA polymerase which recognizes the promoter driving transcription of the protein-encoding gene must be provided to the host cell (U.S. Patent 6,218,145).

Most DNA-dependent RNA polymerases read double-stranded DNA, limiting RNA synthesis to systems in which a double-stranded DNA template is available. The synthesis of RNA using single-stranded DNA is not as common. Synthesizing RNA using a single-stranded DNA template immobilized on a solid support is described in U.S. Patent 5,700,667.

Therefore, this invention provides an RNA polymerase that reads single-stranded DNA. Also provided is an RNA polymerase for which the promoter sequence is present upstream of the transcription initiation site and therefore is not transcribed by the polymerase.

## SUMMARY OF THE INVENTION

The invention provides a novel N4 virion RNA polymerase (vRNAP) and a mini-vRNA polymerase and method of use thereof. The novel polymerases are described by an isolated nucleic acid comprising a region encoding a polypeptide having the amino sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:15. The nucleic acid may comprise the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:14. The vRNAP and mini-vRNA polymerase transcribe nucleic acid operatively linked to an N4 promoter such as a P2 promoter of SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:27, SEQ ID NO:28 or SEQ ID NO:29. The promoter of SEQ ID NO:16 or SEQ ID NO:28 is preferred.

An aspect of the current invention comprises a recombinant host cell comprising a DNA segment encoding a N4 virion RNA polymerase. The DNA segment is either single- or double-stranded and the polypeptide encoded by the DNA segment is preferably SEQ ID NO:4 or SEQ

ID NO:6. The recombinant host cell may be an *E. coli* cell. Another aspect of the current invention comprises a recombinant vector comprising a DNA segment encoding a N4 virion RNA polymerase polypeptide under the control of a promoter.

Yet another aspect of the current invention comprises an isolated polynucleotide comprising a sequence identical or complementary to at least 14 contiguous nucleotides of SEQ ID NO:1. The polynucleotide may comprise at least 20, 25, 30, 35, 40, 45, 50, 60, 75, 100, 150, 200, 250, 300, 400, 600, 800, 1000, 2000, 3000, 3300 or more contiguous nucleotides of SEQ ID NO:1. The polynucleotide may comprise all contiguous nucleotides of SEQ ID NO:3 or all contiguous nucleotides of SEQ ID NO:1.
Similarly, the polynucleotide may comprise at least 20, 25, 30, 35, 40, 45, 50, 60, 75, 100, 150, 200, 250, 300, 400, 600, 800, 1000, 2000, 3000, 3300 or more nucleotides complementary to at least 20, 25, 30, 35, 40, 45, 50, 60, 75, 100, 150, 200, 250, 300, 400, 600, 800, 1000, 2000, 3000, 3300 or more contiguous nucleotides of SEQ ID NO:1.

Another aspect of the current invention comprises a purified N4 virion RNA polymerase comprising at least 20 contiguous amino acids of SEQ ID NO:2. It is preferred that the polymerase contain at least 25, 30, 35, 40, 45, 50, 60, 75, 100, 150, 200, 250, 300, 400, 600, 800, 1000 or more contiguous amino acids of SEQ ID NO:2.

Yet another aspect of the current invention comprises an isolated nucleic acid comprising a region encoding a polypeptide comprising at least 6 contiguous amino acids of SEQ ID NO:2, wherein the polypeptide has RNA polymerase activity under appropriate reaction conditions. It is preferred that this polypeptide comprises at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 75, 100, 150, 200, 250, 300, 400, 600, 800, 1000 or more contiguous amino acids of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:15. The encoded polypeptide may have at least one hexahistidine tag or other tag. The polypeptide may be a mutant of the peptide found in SEQ ID NO:2 or SEQ ID NO:4, such as an enzyme possessing an amino acid substitution at position Y678.

An embodiment of the current invention comprises a method of making RNA. This method comprises: (a) obtaining a N4 virion RNA polymerase (*i.e.* the polypeptide); (b) obtaining DNA wherein the DNA preferably contains a N4 virion RNA polymerase promoter sequence; (c) admixing the RNA polymerase and the DNA; and (d) culturing the RNA

polymerase and the DNA under conditions effective to allow RNA synthesis. Optionally, the method may comprise synthesizing polynucleotides containing modified ribonucleotides or deoxyribonucleotides. The DNA is preferably single-stranded DNA or denatured double-stranded DNA Step (c) may occur in a host cell such as an *E. coli* host cell.

The amino acid sequence of the RNA polymerase is preferably the sequence essentially as set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:15, or a mutant form of the polymerase of SEQ ID NO:4 or SEQ ID NO:6. The mutation may be, for example, at position number Y678 . The RNA transcript may contain derivatized nucleotides.

An aspect of the current invention comprises using an N4 vRNAP promoter to direct transcription. The promoter is preferentially an N4 promoter set forth in SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:27, SEQ ID NO:28 or SEQ ID NO:29. The P2 promoter of SEQ ID NO:16 or SEQ ID NO:28 is preferred. The promoter sequence may be upstream of the transcription initiation site. The promoter may comprise a set of inverted repeats forming a hairpin with a 2-7 base pair long stem and 3-5 base loop having purines in the central and/ or next to the central position of the loop.

The preferred conditions of the transcription method claimed herein includes a pH in step (c) of between 6 and 9, with a pH of between 7.5 and 8.5 more preferred. $Mg^{+2}$ or $Mn^{+2}$ , preferably $Mg^{+2}$ may be admixed. Preferred temperatures for the reaction are 25°C to 50°C with the range of 30°C to 45°C being more preferred and the range of 32°C to 42°C being most preferred. The admixing may occur *in vivo* or *in vitro*.

An aspect of the current invention also includes translation of the RNA after transcription. A reporter gene such as an α-peptide of β-galactosidase may be used. It is preferred the transcription comprises admixing an *E. coli* single-stranded binding protein (*Eco*SSB), a SSB protein homologous to *Eco*SSB or another naturally occurring or chimeric SSB protein homologous to *Eco*SSB with the polymerase and DNA.

The DNA admixed with the RNA polymerase of the current invention may be single-stranded linear DNA or single-stranded circular DNA such as bacteriophage M13 DNA. The DNA may be denatured DNA, such as single-stranded, double-stranded linear or double-stranded

circular denatured DNA. The DNA may also be double-stranded DNA under certain conditions. The RNA may be pure RNA or may contain modified nucleotides. Mixed RNA-DNA oligonucleotides may also be synthesized with the Y678F mutant mini-vRNAP (SEQ ID NO:8) of the current invention.

Yet another aspect of the current invention is the transcription method in which no *Eco*SSB is admixed with the RNA polymerase and DNA; the product of this method is a DNA/RNA hybrid.

The synthesized RNA may comprise a detectable label such as a fluorescent tag, biotin, digoxigenin, 2'-fluoro nucleoside triphosphate, or a radiolabel such as a $^{35}$S- or $^{32}$P-label. The synthesized RNA may be adapted for use as a probe for blotting experiments or in-situ hybridization. Nucleoside triphosphates (NTPs) or derivatized NTPs may be incorporated into the RNA, and may optionally have a detectable label. Deoxynucleoside triphosphates may be incorporated into the RNA.

The RNA may be adapted for use for NMR structural determination. Short RNAs such as those between 10 and 1000 bases or between 10 and 300 bases may be used. The RNA may be adapted for use in spliceosome assembly, splicing reactions or antisense experiments. Also, the RNA may be adapted for use in probing for a complementary nucleotide sequence or for use as a probe in RNase protection studies.

Yet another aspect of the current invention comprises delivering RNA into a cell after transcription of the RNA. The delivery may be by microinjection. Another aspect of the invention comprises amplifying the RNA after transcription.

Another embodiment of the current invention comprises a method of making RNA comprising: (a) obtaining a N4 virion RNA polymerase; (b) obtaining a single-stranded DNA oligonucleotide wherein the oligonucleotide contains a N4 virion RNA polymerase promoter sequence; (c) admixing the RNA polymerase and the oligonucleotide; and (d) culturing the RNA polymerase and the oligonucleotide under conditions effective to allow RNA synthesis. The polymerase preferentially has the amino sequence set forth in SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8. In this embodiment, it is preferred that the DNA has between 20 and 200 bases.

Yet another embodiment of the invention comprises a method of making RNA comprising: (a) obtaining a N4 virion RNA polymerase; (b) obtaining a single-stranded DNA wherein the DNA contains a N4 virion RNA polymerase promoter sequence; (c) obtaining a ribonucleoside triphosphate (XTP) or a derivatized ribonucleoside triphosphate; (d) admixing the RNA polymerase, the DNA and the XTP; and (e) culturing the RNA polymerase and the oligonucleotide under conditions effective to allow RNA synthesis wherein the RNA is a derivatized RNA. The RNA polymerase preferentially has the amino sequence set forth in SEQ ID NO:4 or SEQ ID NO:6 or a mutant of the polymerase of SEQ ID NO:4 or SEQ ID NO:6, such as a mutant with a mutation at position number Y678 or the polymerase of SEQ ID NO:8.

Another embodiment of the invention comprises a method for *in vivo* or *in vitro* protein synthesis comprising: (a) obtaining an RNA polymerase having the amino sequence set forth in SEQ ID NO:4, SEQ ID NO:6 or a mutant thereof; (b) obtaining DNA wherein the DNA contains a N4 virion RNA polymerase promoter sequence; (c) admixing the RNA polymerase and the DNA; (d) culturing the RNA polymerase and the DNA under conditions effective to allow RNA synthesis; and (e) culturing the RNA *in vivo* or *in vitro* under conditions effective to allow protein synthesis. Step (e) may comprise using a two plasmid system or a one plasmid system in which a reporter gene and the RNA polymerase gene are located on the same plasmid.

Yet another embodiment of the invention comprises a method of making a N4 mini-vRNAP comprising: (a) expressing vRNAP, wherein the vRNAP has the amino sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:15 or a mutant thereof; and (b) purifying the vRNAP. The expression of vRNAP may occur in a bacteria, yeast, CHO, Cos, HeLa, NIH3T3, Jurkat, 293, Saos, or a PC12 host cell. A promoter such as pBAD may be used for making the vRNAP in bacterial cells. Any other promoter appropriate to the host cell line used can be employed when expressing vRNAP in other host cells. The polymerase may have a specific recombinant sequence that can be used in purification of the polymerase. The vRNAP may have at least one hexahistidine, FLAG, hemaglutinin or c-myc tag, or may not have a tag.

## BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by

reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1** - Bacteriophage N4 vRNAP promoters on single-stranded templates. These promoters are characterized by conserved sequences and a 5 bp stem, 3 base loop hairpin structure.

**FIG. 2A and FIG. 2B** - N4 vRNAP and generation of mini-vRNAP. FIG. 2A shows a schematic of the N4 vRNAP protein with three motifs: the **T/DxxGR** motif found in DNA-dependent polymerases, the P-loop, an ATP/GTP-binding motif present in some nucleotide-binding proteins, and motif B ($Rx_3Kx_{6-7}YG$), one of three motifs common to the Pol I and Pol $\alpha$ DNA polymerases and the T7-like RNA polymerases. FIG. 2B shows the mini-vRNAP.

**FIG. 3A and FIG. 3B** - Identification of the minimal transcriptionally active domain of N4 vRNAP by proteolytic cleavage. FIG. 3A, SDS-PAGE analysis of the products of vRNAP digestion with trypsin. FIG. 3B N-terminal sequencing of the three initial proteolytic fragments indicated that the stable active polypeptide (mini-vRNAP) corresponds to the middle 1/3 of vRNAP, the region containing the three motifs described in FIG. 2A.

**FIG. 4** - ORFs for full length polymerase, mini-vRNAP and mutants thereof were cloned under pBAD control with an N-terminal hexahistidine tag.

**FIG. 5** - Purification of cloned vRNAP and mini-vRNAP. The left hand side shows the relative amounts of full size and mini-vRNAP proteins purified on TALON columns from the same volume of induced cells. Further concentration on a monoQ column reveals that, in contrast to full size vRNAP, mini-vRNAP is stable after induction (right).

**FIG. 6** - Activation of N4 vRNAP transcription by *Eco*SSB at three different ssDNA concentrations. The extent of *Eco*SSB activation is template-concentration dependent, with highest activation at low DNA template concentration.

**FIG. 7A, FIG. 7B, FIG. 7C, and FIG. 7D** - Effect of *Eco*SSB on ssDNA template recycling. In the absence of *Eco*SSB, no increase in transcription was observed beyond 10 min of incubation (FIG. 7A). Addition of template at 20 min to the reaction carried out in the absence

of *Eco*SSB led to a dramatic increase in RNA synthesis (FIG 7B). RNA synthesis increased linearly throughout the period of incubation (FIG. 7C). Addition of *Eco*SSB at 20 min led to a slow rate of transcriptional recovery (FIG. 7D).

**FIG. 8** - Effect of *Eco*SSB on the state of template DNA and product RNA in vRNAP transcription. Native gel electrophoresis was carried out in the absence and in the presence of *Eco*SSB. Transcription was performed at an intermediate (5 nM) DNA concentration, at which only a 2-fold effect of *Eco*SSB is observed. Either $^{32}$P-labeled template (right panel) or labeled NTPs (left panel) were used to analyze the state of the template (right panel) or RNA product (left panel) in the absence or presence of *Eco*SSB.

**FIG. 9A, FIG. 9B, and FIG. 9C** - Transcription initiation by vRNAP and mini-vRNAP. The initiation properties of the full length and mini-vRNA polymerases were compared at similar molar concentrations (FIG. 9A) using the catalytic autolabeling assay and two reaction conditions: using a template containing +1C, the benzaldehyde derivative of GTP and $\alpha^{32}$P-ATP, or a template containing +1T, the benzaldehyde derivative of ATP and $\alpha^{32}$P-GTP. Comparison of the results in FIGS. 9B and 9C demonstrates that mini-vRNAP exhibits initiation properties similar to full size vRNAP.

**FIG. 10** - Effect of *Eco*SSB on transcription of vRNAP and mini-vRNAP. The elongation and termination properties of vRNAP and mini-vRNAP are compared.

**FIG. 11A and FIG. 11B** - Determination of mini-vRNAP promoter contacts. A 20-base oligonucleotide containing wild type promoter P2 sequence binds with a 1 nM Kd (FIG. 11A). Most oligonucleotides substituted with 5-Iodo-dU at specific positions showed close to wild type affinity except for the oligonucleotides substituted at positions −11 (at the center of the loop) and −8, indicating that these positions are essential for promoter recognition (FIG. 11B). UV crosslinking indicates that mini-vRNAP primarily contacts the −11 position.

**FIG. 12** - Binding affinities of stem-length promoter mutants. Wild type promoter P2 with a 5 bp stem has a Kd of 1nM (top). The stem was shortened by removal of 3' bases (left). The stem can be shortened by two base pairs without change in the binding affinity. The effect

of lengthening the stem by addition of 3' bases is shown (right). The stem can be lengthened by two base pairs without change in the binding affinity

**FIG. 13A and FIG. 13B** - Identification of the transcription start site by catalytic autolabeling. A series of templates were constructed with a single C placed at different distances from the center of the hairpin (position –11) by addition or deletion of the tract of As present at promoter P2 (FIG. 13A). The affinity of mini-vRNAP for these promoters was measured by filter binding, and transcription initiation was measured by catalytic autolabeling of mini-vRNAP. All templates showed similar binding affinities. However, only the template with a C positioned 12 bases downstream from the center of the hairpin was able to support transcription initiation (FIG. 13B).

**FIG. 14** - UV crosslinking of mutant mini-vRNAPases to promoter oligonucleotides. Two mutants (K670A and Y678F) were tested for their ability to bind to wild type promoters. Both mutant RNA polymerases bound to promoter DNA with wild type affinities and crosslinked to 5-Iodo-dU substituted P2 DNA templates at positions –11 and +3 as well as the wild type enzyme, indicating that these polymerase mutations do not affect promoter binding.

**FIG. 15** - Run-off transcription by mutant mini-vRNAPases. The wild type and Y678F (SEQ ID NO:8) enzymes displayed similar activities at both template excess and template-limiting conditions, while the K670A enzyme exhibited decreased activity under both conditions. Under limiting template conditions, all three enzymes were activated by *Eco*SSB (right panel). However, the Y678F enzyme showed reduced discrimination between incorporation of ribo- and deoxyribonucleoside triphosphates.

**FIG. 16** - Mutant mini-vRNAPases in transcription initiation. The initiation properties of the three enzymes were compared using catalytic autolabeling. The K670A enzyme displays significantly reduced activity with the GTP derivative. The Y678F enzyme, in contrast to wild type polymerase, incorporates dATP as efficiently as rATP in a single round of phosphodiester bond formation.

**FIG. 17A, FIG. 17B, and FIG. 17C** - Detection of *in vivo* activities of N4 vRNAP and mini-vRNAP. Transcription of ß-galactosidase $\alpha$-peptide by full size and mini-vRNAP was assayed on inducing-Xgal media (FIG. 17A). Plasmid (pACYC) templates were constructed

with a reporter gene (α-peptide of ß-galactosidase) under the control of vRNAP promoter P2 cloned in either of two orientations (FIG. 17B). Induction of mini-vRNAP led to production and accumulation of detectable levels of the protein, whereas full-length vRNAP was degraded (FIG. 17C).

## DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention overcomes deficiencies in the art by providing a stable RNA polymerase that uses single-stranded DNA and provides a unique system to synthesize RNAs of a desired sequence. The RNA polymerase and mini-vRNA polymerase can be used to synthesize RNA for use as probes in RNase protection studies of DNAs or RNAs, in situ hybridization studies, and in Southern and Northern blot analysis, for the synthesis of defined RNA:DNA hybrids, for NMR structure determination of RNAs, for *in vitro* studies of spliceosome assembly, splicing reactions and antisense experiments, for *in vitro* translation or microinjection, and for nucleic acid amplification. The present invention allows for the synthesis of derivatized RNA and can use ssDNA in the form of single-stranded oligonucleotides, denatured DNA or DNA cloned into M13 templates.

### I. RNA Polymerases

#### *a. Structure and Promoter Recognition of DNA-Dependent RNA Polymerases*

Inspection of the sequences of phage, archaebacterial, eubacterial, eukaryotic and viral DNA-dependent RNA polymerases has revealed the existence of two enzyme families. The eubacterial, eukaryotic, archaebacterial, chloroplast and the vaccinia virus RNA polymerases are complex multisubunit enzymes (5-14 subunits) composed of two large subunits, one to several subunits of intermediate molecular weight (30- 50-kDa) and none to several subunits of small molecular weight (<30-kDa) (Archambault, *et al.*, 1993; Record, *et al.*, 1995). Eubacterial RNA polymerases are the simplest with an $\alpha_2\beta\beta'$ core structure. Sequence comparison of the genes coding for the different subunits of these enzymes has revealed: 1- sequence homology in eight segments (A to H) between β' and the largest subunit of other RNA polymerases, 2- sequence homology in nine segments (A to I) between β and the next largest subunit of other RNA polymerases, 3- sequence homology in 3 segments (1.1, 1.2 and 2) between α and a subunit in RNA polymerases I, II and III (Puhler, *et al.*, 1989; Sweetser, *et al.*, 1987). Not surprisingly, the crystal structures of yeast RNAP II and *E. coli* RNAP core revealed remarkable similarities (Zhang, *et al.*, 1999; Cramer, *et al.*, 2001).

In contrast, members of the phage T7-like (T3, SP6) family of RNA polymerases consist of a single ( ~100 kDa) polypeptide which catalyzes all functions required for accurate transcription (Cheetham, *et al.*, 2000). The heterodimeric bacteriophage N4 RNAP II, nuclear-coded mitochondrial, and *Arabidopsis* chloroplast RNA polymerases show sequence similarity to the phage RNA polymerases (Cermakian, *et al.*, 1996; Hedtke, *et al.*, 1997; Zehring, *et al.*, 1983). Three sequence motifs -A and C, which contain the two aspartic acids required for catalysis, and motif B- are conserved in polymerases that use DNA as a template (Delarue, *et al.*, 1990). The crystal structure of T7 RNAP resembles a "cupped right hand" with "palm," fingers" and "thumb" subdomains (Sousa, *et al.*, 1993). The two catalytic aspartates are present in the "palm" of the structure. This structure is shared by the polymerase domains of *E. coli* DNA polymerase I and HIV reverse transcriptase (Sousa, 1996). Genetic, biochemical and structural information indicates that T7 RNA polymerase contains additional structures dedicated to nascent RNA binding, promoter recognition, dsDNA unwinding and RNA:DNA hybrid unwinding (Cheetham, *et al.*, 2000; Sousa, 1996)

Both Class I and Class II RNA polymerases recognize specific sequences, called promoters, on B form double-stranded DNA. Eubacterial promoters (except those recognized by $\sigma^{54}$) are characterized by two regions of sequence homology: the -10 and the -35 hexamers (Gross, *et al.*, 1998). Specificity of promoter recognition is conferred to the core enzyme by the $\sigma$ subunit, which makes specific interactions with the –10 and –35 sequences through two distinct DNA binding domains (Gross, *et al.*, 1998). This modular promoter structure is also present at the promoters for eukaryotic RNA polymerases I, II and III. Transcription factors TFIIIA and TFIIIC direct recognition of RNAP III to two separate sequences (boxes A and C, separated by defined spacing) at the 5S gene promoter, while transcription factors TFIIIB and TFIIIC direct recognition of this enzyme to blocks A and B, separated by variable distance (31-74 bp) at the tRNA promoters (Paule, *et al.*, 2000). Sequences important for RNAP I transcription initiation at the human rRNA promoters are also restricted to two regions: the "core" region located at -40 to +1 and the "upstream" region present at -160 to –107 (Paule, *et al.*, 2000). Assembly of the initiation complex at RNAP II promoters requires several general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH). Recognition involves three core elements: the TATA box located at position –30 and recognized by TBP, the initiator element located near -1, and the downstream promoter element near +30 (Roeder, 1996).

Promoters for the T7-like and mitochondrial RNAPases are simpler. The T7-type RNAP promoters span a continuous highly conserved 23 bp region extending from position -17 to +6 relative to the start site of transcription (+1) (Rong, *et al.*, 1998). The yeast mitochondrial RNAP promoters are even smaller, extending from -8 to +1 (Shadel, *et al.*, 1993). One exception are the promoters for N4 RNAP II, which are restricted to two blocks of conserved sequence: a/tTTTA at +1 and AAGACCTG present 18-26 bp upstream of +1 (Abravaya, *et al.*, 1990).

The activity of the multisubunit class of RNA polymerases is enhanced by activators at weak promoters. Transcription activators generally bind at specific sites on double-stranded DNA upstream of the -35 region (with the exception of the T4 sliding clamp activator), or at large distances in the cases of enhancers (Sanders, *et al.*, 1997). Activators modulate transcription by increasing the binding (formation of closed complex) or isomerization (formation of open complex) steps of transcription through interactions with the $\alpha$ or $\sigma$ subunits of RNAP (Hochschild, *et al.*, 1998). An exception is N4SSB, the activator of *E. coli* RNAP$\sigma^{70}$ at the bacteriophage N4 late promoters, which activates transcription through direct interactions with the $\beta'$ subunit of RNAP in the absence of DNA binding (Miller, *et al.*, 1997).

Proteins that bind to ssDNAs with high affinity but without sequence specificity have been purified and characterized from several prokaryotes, eukaryotes, and their viruses (Chase, *et al.*, 1986). These proteins (SSBs), which are required for replication, recombination and repair, bind stoichiometrically and, in many cases, cooperatively to ssDNA to cover the transient single-stranded regions of DNA that normally arise *in vivo* as a result of replication, repair and recombination. Binding to DNA results in the removal of hairpin structures found on ssDNA, providing an extended conformation for proteins involved in DNA metabolism. Several lines of evidence suggest that single-stranded DNA binding proteins play a more dynamic role in cellular processes. Genetic and biochemical evidence indicates that these proteins are involved in a multitude of protein-protein interactions including transcription activation (Rothman-Denes, *et al.*, 1999).

### *b. The Bacteriophage N4 Virion RNA Polymerase*

Bacteriophage N4 virion RNA polymerase (N4 vRNAP) is present in N4 virions and is injected into the *E. coli* cell at the beginning of infection, where it is responsible for transcription of the N4 early genes (Falco, *et al.*, 1977; Falco, *et al.*, 1979; Malone, *et al.*, 1988). The N4 vRNAP gene maps to the late region of the N4 genome (Zivin, *et al.*, 1981). N4 vRNAP purified from virions is composed of a single polypeptide with an apparent molecular mass of approximately 320,000 kDa (Falco, *et al.*, 1980). In contrast to other DNA-dependent RNAPases, N4 vRNAP recognizes promoters on single-stranded templates (Falco, *et al.*, 1978). These promoters are characterized by conserved sequences and a 5 bp stem, 3 base loop hairpin structure (**FIG. 1**) (Haynes, *et al.*, 1985; Glucksmann, *et al.*, 1992). *In vivo, E. coli* gyrase and single-stranded binding protein are required for transcription by N4 vRNAP (Falco, *et al.*, 1980; Markiewicz, *et al.*, 1992).

Sequencing of the N4 vRNAP gene revealed an ORF coding for a protein 3,500 amino acids in length (SEQ ID NO:1-2). Inspection of the sequence revealed no extensive homology to either the multisubunit or the T7-like families of RNA polymerases. However, three motifs are present (FIG. 2A): the T/DxxGR motif found in DNA-dependent polymerases, and Motif B ($Rx_3Kx_{6-7}YG$), one of three motifs common to the Pol I and Pol $\alpha$ DNA polymerases and the T7-like RNA polymerases.

### *c. Transcription Using N4 vRNAP*

RNA synthesis requires RNA polymerase, a DNA template, an activated precursor (the ribonucleoside triphosphates ATP, GTP, UTP and CTP (XTP)), and divalent metal ions such as $Mg^{2+}$ or $Mn^{2+}$. The metal ion $Mg^{2+}$ is strongly preferred. Synthesis of RNA begins at the promoter site on the DNA. This site contains a sequence which the RNA polymerase recognizes and binds. The RNA synthesis proceeds until a termination site is reached. N4 vRNAP termination signals comprise a hairpin loop that forms in the newly synthesized RNA which is followed by a string of uracils (poly U). The sequence of the terminator signals for vRNAP present in the N4 genome include SEQ ID NOS: 21 - 26. These N4 vRNAP termination signals possess all of the characteristics of eubacterial sequence-dependent terminators.

The ribonucleoside triphosphate may be derivatized with, for example, biotin. Derivatized XTPs can be used for the preparation of derivatized RNA. Exemplary methods for

making derivatized XTPs are disclosed in detail in Rashtchian *et al.* (1992), herein incorporated by reference.

Single-stranded DNA of varying lengths can be used as a template for RNA synthesis using the N4 vRNAP or mini-vRNAP. Oligonucleotides and polynucleotides of intermediate length may be used. One particular single-stranded DNA that may be used is M13 DNA. M13 genomic DNA exists temporarily inside infected *E. coli* cells as a double-stranded DNA plasmid and is packaged as a small, single-stranded circular DNA into phage particles. M13 phage particles are secreted by an infected cell and single-stranded DNA can be purified from these particles for use as a transcription template. Initially M13 phage vectors required a working knowledge of phage biology and were primarily used for creating single-strand DNA molecules for DNA sequencing. M13-derived cloning vectors called "phagemids" take advantage of M13 replication to produce single-strand molecules, but can be propagated as conventional ColE1-based replicating double-strand plasmids.

*Eco*SSB is essential for N4 vRNAP transcription *in vivo* (Falco *et al*, 1978; Glucksmann, *et al.*, 1992, herein incorporated by reference). *Eco*SSB is a specific activator of N4 vRNAP on single-stranded and supercoiled double-stranded DNA templates. *Eco*SSB, unlike other SSBs, does not melt the N4 vRNAP promoter hairpin structure (Glucksmann-Kuis, *et al.*, 1996). *Eco*SSB has a high specificity for N4 vRNAP and mini-vRNAP resulting from *Eco*SSB's ability to stabilize the template-strand hairpin, whereas the nontemplate strand hairpin is destabilized. Other single-stranded DNA binding proteins destabilize the template-strand hairpin (Glucksmann-Kuis *et al*, 1996; Dai *et al.*, 1998). When *Eco*SSB is not used in N4 vRNAP transcription *in vitro*, a DNA:RNA hybrid is formed, preventing template reutilization.

## II. Genes and DNA Segments

Important aspects of the present invention concern isolated DNA segments and recombinant vectors encoding N4 vRNAP or more particularly mini-vRNAP or a mutant of mini-vRNAP and the creation and use of recombinant host cells through the application of DNA technology, that express a wild type, polymorphic or mutant vRNAP. Other aspects of the present invention concern isolated nucleic acid segments and recombinant vectors encoding vRNAP. Sequences of SEQ ID NO:1, 3, 5, 7, 14 and biologically functional equivalents thereof are used in the current invention. Single-stranded DNA oligonucleotides and polynucleotides can be used as DNA templates.

The present invention concerns isolated nucleic acid segments that are capable of expressing a protein, polypeptide or peptide that has RNA polymerase activity. As used herein, the term "nucleic acid segment" refers to a nucleic acid molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a nucleic acid segment encoding vRNAP refers to a nucleic acid segment that contains wild-type, polymorphic or mutant vRNAP coding sequences yet is isolated away from, or purified free from, total bacterial or N4 phage genomic DNA. Included within the term "nucleic acid segment," are nucleic acid segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

Similarly, a nucleic acid segment comprising an isolated or purified vRNAP gene refers to a nucleic acid segment including vRNAP protein, polypeptide or peptide coding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those of skill in the art, this functional term includes both genomic sequences, cDNA sequences and engineered segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, vRNAPs and mutants of vRNAP encoding sequences.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case the vRNAP, or more particularly mini-vRNAP genes, forms the significant part of the coding region of the nucleic acid segment, and that the nucleic acid segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

The term "a sequence essentially as set forth in SEQ ID NO:2 means, for example, that the sequence substantially corresponds to a portion of SEQ ID NO:2 and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids of SEQ ID NO:2. This applies with respect to all peptide and protein sequences herein, such as those of SEQ ID NO:4, 6, 8 and 15.

The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein. Accordingly, sequences that have about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or about 99%, and any range derivable therein, such as, for example, about 70% to about 80%, and more preferably about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:2 will be sequences that are "essentially as set forth in SEQ ID NO:2, provided the biological activity of the protein is maintained. In particular embodiments, the biological activity of a vRNAP protein, polypeptide or peptide, or a biologically functional equivalent, comprises transcription. A preferred transcriptional activity that may be possessed by a vRNAP protein, polypeptide or peptide, or a biologically functional equivalent, is RNA synthesis using single-stranded N4 vRNAP promoter-containing DNA as a template.

In certain other embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors that include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:1. The term "essentially as set forth in SEQ ID NO:1 is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:1 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:1. Again, nucleic acid segments that encode proteins, polypeptide or peptides exhibiting RNAP activity will be most preferred.

The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine and serine, and also refers to codons that encode biologically equivalent amino acids. For optimization of expression of vRNAP in human cells, the codons are shown in Table 1 in preference of use from left to right. Thus, the most preferred codon for alanine is thus "GCC," and the least is "GCG" (see Table 1 below). Codon usage for various organisms and organelles can be found at the website http://www.kazusa.or.jp/codon/, incorporated herein by reference, allowing one of skill in the art to optimize codon usage for expression in various organisms using the disclosures herein. Thus, it is contemplated that codon usage may be optimized for other animals, as well as other organisms such as a prokaryote (*e.g.*, an eubacteria), an archaea, an eukaryote (*e.g.*, a protist, a

plant, a fungus, an animal), a virus and the like, as well as organelles that contain nucleic acids, such as mitochondria or chloroplasts, based on the preferred codon usage as would be known to those of ordinary skill in the art.

| Table 1-Preferred Human DNA Codons | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Amino Acids** | | | | **Codons** | | | | | |
| Alanine | Ala | A | | GCC | GCT | GCA | GCG | | |
| Cysteine | Cys | C | | TGC | TGT | | | | |
| Aspartic acid | Asp | D | | GAC | GAT | | | | |
| Glutamic acid | Glu | E | | GAG | GAA | | | | |
| Phenylalanine | Phe | F | | TTC | TTT | | | | |
| Glycine | Gly | G | | GGC | GGG | GGA | GGT | | |
| Histidine | His | H | | CAC | CAT | | | | |
| Isoleucine | Ile | I | | ATC | ATT | ATA | | | |
| Lysine | Lys | K | | AAG | AAA | | | | |
| Leucine | Leu | L | | CTG | CTC | TTG | CTT | CTA | TTA |
| Methionine | Met | M | | ATG | | | | | |
| Asparagine | Asn | N | | AAC | AAT | | | | |
| Proline | Pro | P | | CCC | CCT | CCA | CCG | | |
| Glutamine | Gln | Q | | CAG | CAA | | | | |
| Arginine | Arg | R | | CGC | AGG | CGG | AGA | CGA | CGT |
| Serine | Ser | S | | AGC | TCC | TCT | AGT | TCA | TCG |
| Threonine | Thr | T | | ACC | ACA | ACT | ACG | | |
| Valine | Val | V | | GTG | GTC | GTT | GTA | | |
| Tryptophan | Trp | W | | TGG | | | | | |
| Tyrosine | Tyr | Y | | TAC | TAT | | | | |

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein, polypeptide or peptide activity. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5'

or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

Excepting intronic or flanking regions, and allowing for the degeneracy of the genetic code, sequences that have about 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or about 99%, and any range derivable therein, such as, for example, about 50% to about 80%, and more preferably about 81% and about 90%; or even more preferably, between about 91% and about 99%; of nucleotides that are identical to the nucleotides of SEQ ID NO:1 will be sequences that are "essentially as set forth in SEQ ID NO:1".

***a. Nucleic Acid Hybridization***

The nucleic acid sequences disclosed herein also have a variety of uses. Contiguous sequences from vRNAP nucleic acid sequences can be used, for example, as templates to synthesize vRNAP.

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:1, 3, 5, 7 and 14. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementary rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:1 under stringent conditions such as those described herein.

As used herein, a "DNA/RNA hybrid" is understood to mean that a single strand of RNA is hybridized to a single strand of DNA.

The term "appropriate reaction conditions" as described herein mean that temperature, pH, buffer, and other parameters are adjusted to optimize the reaction rate and yield.

As used herein, "hybridization," "hybridizes" or "capable of hybridizing" is understood to mean the forming of a double or triple stranded molecule or a molecule with partial double or triple stranded nature. The term "hybridization," "hybridize(s)" or "capable of hybridizing" encompasses the terms "stringent condition(s)" or "high stringency" and the terms "low stringency" or "low stringency condition(s)."

As used herein "stringent condition(s)" or "high stringency" are those conditions that allow hybridization between or within one or more nucleic acid strand(s) containing complementary sequence(s), but precludes hybridization of random sequences. Stringent conditions tolerate little, if any, mismatch between a nucleic acid and a target strand. Such conditions are well known to those of ordinary skill in the art, and are preferred for applications requiring high selectivity. Non-limiting applications include isolating a nucleic acid, such as a gene or a nucleic acid segment thereof, or detecting at least one specific mRNA transcript or a nucleic acid segment thereof, and the like.

Stringent conditions may comprise low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. It is understood that the temperature and ionic strength of a desired stringency are determined in part by the length of the particular nucleic acid(s), the length and nucleobase content of the target sequence(s), the charge composition of the nucleic acid(s), and to the presence or concentration of formamide, tetramethylammonium chloride or other solvent(s) in a hybridization mixture.

It is also understood that these ranges, compositions and conditions for hybridization are mentioned by way of non-limiting examples only, and that the desired stringency for a particular hybridization reaction is often determined empirically by comparison to one or more positive or negative controls. Depending on the application envisioned it is preferred to employ varying conditions of hybridization to achieve varying degrees of selectivity of a nucleic acid towards a target sequence. In a non-limiting example, identification or isolation of a related target nucleic acid that does not hybridize to a nucleic acid under stringent conditions may be achieved by hybridization at low temperature and/or high ionic strength. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C. Under these conditions, hybridization may occur even though the sequences of probe and target strand are not perfectly complementary, but are mismatched at one or more positions. In another example, a low stringency condition could be provided by about 0.15 M to about 0.9 M salt,

at temperatures ranging from about 20°C to about 55°C. Of course, it is within the skill of one in the art to further modify the low or high stringency conditions to suit a particular application. For example, in other embodiments, hybridization may be achieved under conditions of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, at temperatures ranging from approximately 40°C to about 72°C.

Accordingly, the nucleotide sequences of the disclosure may be used for their ability to selectively form duplex molecules with complementary stretches of genes or RNAs or to provide primers for amplification of DNA or RNA from tissues. Depending on the application envisioned, it is preferred to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, enhancers, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

For example, nucleic acid fragments may be prepared that include a contiguous stretch of nucleotides identical to or complementary to SEQ ID NO:1, 3, 5, 7 or 14. Nucleic acid fragments for use as a DNA transcription template may also be prepared. These fragments may be short or of intermediate lengths, such as, for example, about 8, about 10 to about 14, or about 15 to about 20 nucleotides, and that are chromosome-sized pieces, up to about 35,000, about 30,000, about 25,000, about 20,000, about 15,000, about 10,000, or about 5,000 base pairs in length, as well as DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths of these lengths listed above, *i.e.*, any range derivable therein and any integer derivable therein such a range) are also contemplated to be useful.

For example, it will be readily understood that "intermediate lengths," in these contexts, means any length between the quoted ranges, such as 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 130, 140, 150, 160, 170, 180, 190, including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002, 15,000, 20,000 and the like.

Various nucleic acid segments may be designed based on a particular nucleic acid sequence, and may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, *etc.*, an algorithm defining all nucleic acid segments can be created:

$$n \text{ to } n + y$$

where n is an integer from 1 to the last number of the sequence and y is the length of the nucleic acid segment minus one, where n + y does not exceed the last number of the sequence. Thus, for a 10-mer, the nucleic acid segments correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and/or so on. For a 15-mer, the nucleic acid segments correspond to bases 1 to 15, 2 to 16, 3 to 17 ... and/or so on. For a 20-mer, the nucleic segments correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and/or so on. In certain embodiments, the nucleic acid segment may be a probe or primer. As used herein, a "probe" generally refers to a nucleic acid used in a detection method or composition. As used herein, a "primer" generally refers to a nucleic acid used in an extension or amplification method or composition.

The use of a hybridization probe of between 17 and 100 nucleotides in length, or in some aspect of the invention even up to 1-2 Kb or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 20 bases in length are generally preferred, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of particular hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having complementary sequences over stretches of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization, as in PCR™, for detection of expression of corresponding

genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface to remove non-specifically bound probe molecules, hybridization is detected, or even quantified, by means of the label.

### *b. Nucleic Acid Amplification*

Nucleic acid used as a template for amplification is isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to nucleic acids are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer," as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty or thirty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label, or even via a system using electrical or thermal impulse signals (Affymax technology).

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR™) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each incorporated herein by reference in its entirety.

Briefly, in PCR™, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products, and the process is repeated.

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641, filed December 21, 1990, incorporated herein by reference. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPA No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, incorporated herein by reference, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to

that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence, which can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification method described in GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction,

heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double-stranded DNA molecules are heat denatured again. In either case, the single-stranded DNA is made fully double-stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into single-stranded DNA, which is then converted to double-stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not

produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, 1990, incorporated herein by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide," thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

### *c. Nucleic Acid Detection*

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention such as all or part of SEQ ID NO:1, 3, 5, 7, 14 or a mutant thereof in combination with an appropriate means, such as a label, for hybridization assays, RNase protection and Northern hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In embodiments wherein nucleic acids are amplified, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.,* 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are

integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols (see Sambrook *et al.,* 1989). Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

Other methods for genetic screening to accurately detect mutations in genomic DNA, cDNA or RNA samples may be employed, depending on the specific situation.

Historically, a number of different methods have been used to detect point mutations, including denaturing gradient gel electrophoresis ("DGGE"), restriction enzyme polymorphism analysis, chemical and enzymatic cleavage methods, and others. The more common procedures currently in use include direct sequencing of target regions amplified by PCR™ (see above) and single-strand conformation polymorphism analysis ("SSCP").

Another method of screening for point mutations is based on RNase cleavage of base pair mismatches in RNA/DNA and RNA/RNA heteroduplexes. As used herein, the term "mismatch" is defined as a region of one or more unpaired or mispaired nucleotides in a double-stranded RNA/RNA, RNA/DNA or DNA/DNA molecule. This definition thus includes mismatches due to insertion/deletion mutations, as well as single and multiple base point mutations.

U.S. Patent No. 4,946,773 describes an RNase A mismatch cleavage assay that involves annealing single-stranded DNA or RNA test samples to an RNA probe, and subsequent treatment of the nucleic acid duplexes with RNase A. After the RNase cleavage reaction, the RNase is inactivated by proteolytic digestion and organic extraction, and the cleavage products are denatured by heating and analyzed by electrophoresis on denaturing polyacrylamide gels. For the detection of mismatches, the single-stranded products of the RNase A treatment, electrophoretically separated according to size, are compared to similarly treated control duplexes. Samples containing smaller fragments (cleavage products) not seen in the control duplex are scored as positive.

Currently available RNase mismatch cleavage assays, including those performed according to U.S. Patent No. 4,946,773, require the use of radiolabeled RNA probes. Myers and Maniatis in U.S. Patent No. 4,946,773 describe the detection of base pair mismatches using RNase A. Other investigators have described the use of an *E. coli* enzyme, RNase I, in mismatch assays. Because it has broader cleavage specificity than RNase A, RNase I would be a desirable enzyme to employ in the detection of base pair mismatches if components can be found to decrease the extent of non-specific cleavage and increase the frequency of cleavage of mismatches. The use of RNase I for mismatch detection is described in literature from Promega Biotech. Promega markets a kit containing RNase I that is shown in their literature to cleave three out of four known mismatches, provided the enzyme level is sufficiently high.

The RNase Protection assay was first used to detect and map the ends of specific mRNA targets in solution. The assay relies on being able to easily generate high specific activity radiolabeled RNA probes complementary to the mRNA of interest by *in vitro* transcription. Originally, the templates for *in vitro* transcription were recombinant plasmids containing bacteriophage promoters. The probes are mixed with total cellular RNA samples to permit hybridization to their complementary targets, then the mixture is treated with RNase to degrade excess unhybridized probe. Also, as originally intended, the RNase used is specific for

single-stranded RNA, so that hybridized double-stranded probe is protected from degradation. After inactivation and removal of the RNase, the protected probe (which is proportional in amount to the amount of target mRNA that was present) is recovered and analyzed on a polyacrylamide gel.

The RNase Protection assay was adapted for detection of single base mutations. In this type of RNase A mismatch cleavage assay, radiolabeled RNA probes transcribed *in vitro* from wild-type sequences are hybridized to complementary target regions derived from test samples. The test target generally comprises DNA (either genomic DNA or DNA amplified by cloning in plasmids or by PCR™), although RNA targets (endogenous mRNA) have occasionally been used. If single nucleotide (or greater) sequence differences occur between the hybridized probe and target, the resulting disruption in Watson-Crick hydrogen bonding at that position ("mismatch") can be recognized and cleaved in some cases by single-strand specific ribonuclease. To date, RNase A has been used almost exclusively for cleavage of single-base mismatches, although RNase I has recently been shown as useful also for mismatch cleavage. There are recent descriptions of using the MutS protein and other DNA-repair enzymes for detection of single-base mismatches.

Nuclease S1 analysis of reaction products can be used to measure RNA. An exemplary procedure for S1 analysis involves hybridization reaction with the RNA of interest (0.005-0.1mg) and an excess of S1 probe which comprises a labeled oligonucleotide complementary to 20-80 or more sequential nucleotides of a specific RNA in S1 hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM Pipes, pH 6.4). After denaturation for 4 min at 94 °C, overnight hybridization at 30°C and precipitation with ethanol, the S1 probe/RNA mixture is resuspended in S1 buffer (0.26 M NaCl, 0.05 M sodium acetate, pH 4.6, and 4.5 mM zinc sulfate). The sample is divided into two volumes and 100 units of S1 nuclease (Sigma Chemical Company) is added to one tube. The samples are incubated for 60 minutes at 37°C; then EDTA (10 mM final concentration) and 15 g polyI-polyC RNA are added and the sample is extracted with phenol/chloroform and precipitated in ethanol. The samples are then subjected to polyacrylamide gel electrophoresis.

One method to produce a radiolabeled RNA probe with high specific activity includes admixing a radiolabeled NTP during transcription. Suitable isotopes for radiolabeling include $^{35}S$- and $^{32}P$-labeled UTP, GTP , CTP or ATP. For optimal results, a gel-purified radiolabeled

RNA probe which is preferentially 300-500 bases in length, with a specific activity of 1-3 X10^8 cpm/µg should be generated using the RNA polymerase of the current invention. In order to produce this *in vitro* transcript, it is often advisable to use a high specific activity (*e.g.*, [α-$^{32}$P]CTP at 3,000Ci/mmol) NTP. To prevent background hybridization, it is important to remove plasmid template DNA by digestion which can be done with, for example, RQ1 RNase-Free DNase followed by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation.

Another method for producing radiolabeled probes includes using a riboprobe system which can produce high specific activity, radiolabeled RNA probes or microgram quantities of *in vitro* transcript. Riboprobes are useful with radiolabeled RNA probes in many applications including RNase protection, Northern hybridization, S1 analysis and in situ hybridization assays. The principle components of an *in vitro* transcription are the riboprobe, an RNA polymerase, a DNA template which includes a phage RNA polymerase promoter and ribonucleotide triphosphates.

### *d. Cloning vRNAP Genes*

The present invention contemplates cloning vRNAP, or more particularly mini-vRNAP genes. A technique often employed by those skilled in the art of protein production today is to obtain a so-called "recombinant" version of the protein, to express it in a recombinant cell and to obtain the protein, polypeptide or peptide from such cells. These techniques are based upon the "cloning" of a nucleic acid molecule encoding the protein from a DNA library, *i.e.*, on obtaining a specific DNA molecule distinct from other portions of DNA. This can be achieved by, for example, cloning a cDNA molecule, or cloning a genomic-like DNA molecule.

The first step in such cloning procedures is the screening of an appropriate DNA library, such as, for example, from a phage, bacteria, yeast, fungus, mouse, rat, monkey or human. The screening protocol may utilize nucleotide segments or probes that are designed to hybridize to cDNA or genomic sequences of vRNAPs from protists. Additionally, antibodies designed to bind to the expressed vRNAP proteins, polypeptides, or peptides may be used as probes to screen an appropriate viral, eubacterial, archaebacterial or eukaryotic DNA expression library. Alternatively, activity assays may be employed. The operation of such screening protocols are well known to those of skill in the art and are described in detail in the scientific literature, for example, in Sambrook *et al.* (1989), incorporated herein by reference. Moreover, as the present invention encompasses the cloning of genomic segments as well as cDNA molecules, it is

contemplated that suitable genomic cloning methods, as known to those in the art, may also be used.

Encompassed by the invention are DNA segments encoding relatively small peptides, such as, for example, peptides of from about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 35, about 40, about 45, to about 50 amino acids in length, and more preferably, of from about 15 to about 30 amino acids in length; as set forth in SEQ ID NO:2, 4, 6, 8 or 15 and also larger polypeptides up to and including proteins corresponding to the full-length sequences set forth in SEQ ID NO:2 and SEQ ID NO:15, and any range derivable therein and any integer derivable in such a range. In addition to the "standard" DNA and RNA nucleotide bases, modified bases are also contemplated for use in particular applications of the present invention. A table of exemplary, but not limiting, modified bases is provided herein below.

**Table 2 Modified Bases**

| Abbr. | Modified base description | Abbr. | Modified base description |
|---|---|---|---|
| ac4c | 4-acetylcytidine | Mam5s2u | 5-methoxyaminomethyl-2-thiouridine |
| chm5u | 5-(carboxyhydroxylmethyl)uridine | Man q | Beta,D-mannosylqueosine |
| Cm | 2′-O-methylcytidine | Mcm5s2u | 5-methoxycarbonylmethyl-2-thiouridine |
| Cmnm5s2u | 5-carboxymethylaminomethyl-2-thioridine | Mcm5u | 5-methoxycarbonylmethyluridine |
| Cmnm5u | 5-carboxymethylaminomethyluridine | Mo5u | 5-methoxyuridine |
| D | Dihydrouridine | Ms2i6a | 2-methylthio-N6-isopentenyladenosine |
| Fm | 2'-O-methylpseudouridine | Ms2t6a | N-((9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine |
| gal q | Beta,D-galactosylqueosine | Mt6a | N-((9-beta-D-ribofuranosylpurine-6-yl) N-methyl-carbamoyl)threonine |
| Gm | 2'-O-methylguanosine | Mv | Uridine-5-oxyacetic acid methylester |
| I | Inosine | o5u | Uridine-5-oxyacetic acid (v) |
| I6a | N6-isopentenyladenosine | Osyw | Wybutoxosine |

**Table 2 Modified Bases**

| Abbr. | Modified base description | Abbr. | Modified base description |
|---|---|---|---|
| m1a | 1-methyladenosine | P | Pseudouridine |
| m1f | 1-methylpseudouridine | Q | Queosine |
| m1g | 1-methylguanosine | s2c | 2-thiocytidine |
| m1I | 1-methylinosine | s2t | 5-methyl-2-thiouridine |
| m22g | 2,2-dimethylguanosine | s2u | 2-thiouridine |
| m2a | 2-methyladenosine | s4u | 4-thiouridine |
| m2g | 2-methylguanosine | T | 5-methyluridine |
| m3c | 3-methylcytidine | t6a | N-((9-beta-D-ribofuranosylpurine-6-yl) carbamoyl)threonine |
| m5c | 5-methylcytidine | Tm | 2'-O-methyl-5-methyluridine |
| m6a | N6-methyladenosine | Um | 2'-O-methyluridine |
| m7g | 7-methylguanosine | Yw | Wybutosine |
| Mam5u | 5-methylaminomethyluridine | X | 3-(3-amino-3-carboxypropyl)uridine, (acp3)u |

## III. Recombinant Vectors, Promoters, Host Cells and Expression

Recombinant vectors form an important further aspect of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a proteinaceous molecule, but it need not be, such as in the case of mini-vRNAP transcribing an RNA using a single-stranded DNA template. Thus, in certain embodiments, expression includes both transcription of a single-stranded DNA and translation of an RNA into the protein product. In other embodiments, expression only includes transcription of the nucleic acid. A recombinant vector can also be used for delivery of the RNA of the current invention.

Particularly useful vectors are contemplated to be those vectors in which the coding portion of the DNA segment, whether encoding a full length protein or smaller polypeptide or peptide, is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the

correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

One particularly useful vector is pBAD. The pBAD expression vectors allow for greater control of bacterial expression of recombinant proteins and allow tight regulation for turning expression on or off. pBAD vectors allow for dose dependent induction for modulation of expression levels. The pBAD expression system helps overcome two of the most common problems of heterologous protein expression in bacteria: toxicity of the recombinant protein to the host and insolubility of the recombinant protein when it is expressed at high, uncontrolled levels. In both cases, a tightly-regulated expression system is critical for maximizing recombinant protein yields. The pBAD expression system is based on the araBAD operon which controls the arabinose metabolic pathway in *E. coli* and allows for precise modulation of heterologous expression to levels that are optimal for recovering high yields of the protein of interest (Guzman *et al.*, 1995).

***a. Promoters***

Any promoters normally found in a host cell in the native state can be used in the present invention to drive expression of N4 vRNA or mini-vRNA polymerase. Also, promoters not normally found in the host cell in the native state that are recognized by a native, normally native host cell RNA polymerase, or non-native RNA polymerase expressed in the cell can be used in the present invention to drive expression of the RNA polymerase. Other promoters may be selected from a nucleic acid sequence database accessible to those of skill in the art, *e.g.*, GenBank, or the promoter can be isolated by a screening method. A promoter recognized by the host cell can be operably linked to the gene or genes encoding the N4 RNA polymerase. The operable linkage can be constructed using any known techniques for DNA manipulation, as referred to herein.

Promoters are described as either constitutive or inducible. Constitutive promoters actively drive expression of genes under their control. Inducible promoters, in contrast, are activated in response to specific environmental stimuli. Both constitutive and inducible promoters can be used in the present invention for expressing non-host genes in a host cell.

Inducible promoters include, but are not limited to, trp, tac, lac, ara, recA, $\lambda$Pr, and $\lambda$Pl. These promoters and others that can be used in the present invention for expression of the N4

vRNA or mini-vRNA polymerase, in embodiments in which the host cell is *E. coli*, are described by Makrides, Microbiological Reviews, (1996), 60, 512-538, herein incorporated by reference. Further, in embodiments of the present invention wherein the host cell is a microbe other than *E. coli*, such as *Saccharomyces*, *Bacillus*, and *Pseudomonas*, any inducible promoter known to those skilled in the art to be active in the host cell can be used to drive expression of the heterologous RNA polymerase. (U. S. Patent No. 6,218,145).

The promoter may be in the form of the promoter that is naturally associated with N4 vRNA or mini-vRNA polymerase, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein (PCR™ technology is disclosed in U.S. Patent 4,683,202 and U.S. Patent 4,682,195, each incorporated herein by reference).

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with N4 vRNA or mini-vRNA polymerase in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, protist, or mammalian cell, and/or promoters made by the hand of man that are not "naturally occurring," *i.e.*, containing different elements from different promoters, or mutations that increase, decrease, or alter expression.

Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins, polypeptides or peptides.

At least one module in a promoter generally functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase promoter, the spacing between promoter elements can be increased to 50 base pairs apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of the instant nucleic acids. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression are contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 3 and 4 below list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of a vRNAP gene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof.

In certain embodiments of the invention, promoter sequences may be used that that are recognized specifically by a DNA-dependent RNA polymerase, such as, but not limited to, those described by Chamberlin and Ryan (1982) and by Jorgensen et al., (1991). These promoters can

be used to express a wild-type or mutant form of a miniV RNA polymerase of the invention. Several RNA polymerase promoter sequences are especially useful, including, but not limited to, promoters derived from SP6 (e.g., Zhou and Doetsch, 1993), T7 (e.g., Martin, and Coleman, 1987) and T3 (e.g., McGraw et al., 1985). An RNA polymerase promoter sequence derived from *Thermus thermophilus* can also be used (see, e.g., Wendt et al., 1990; Faraldo et al., 1992; Hartmann et al., 1987; Hartmann et al., 1991). The length of the promoter sequence will vary depending upon the promoter chosen. For example, the T7 RNA polymerase promoter can be only about 25 bases in length and act as a functional promoter, while other promoter sequences require 50 or more bases to provide a functional promoter.

In other embodiments of the invention, a promoter is used that is recognized by an RNA polymerase from a T7-like bacteriophage. The genetic organization of all T7-like phages that have been examined has been found to be essentially the same as that of T7. Examples of T7-like phages according to the invention include, but are not limited to *Escherichia coli* phages T3, .phi.I, .phi.II, W31, H, Y, A1, 122, cro, C21, C22, and C23; *Pseudomonas putida* phage gh-1; *Salmonella typhimurium* phage SP6; *Serratia marcescens* phages IV; *Citrobacter* phage ViIII; and *Klebsiella* phage No. 11 (Hausmann, 1976; Korsten *et al.*, 1975; Dunn, *et al.* 1971; Towle, *et al.*, 1975; Butler and Chamberlin, 1982).

When a T7 RNA polymerase promoter, or another T7-like RNA polymerase promoter is used to express a wild-type or mutant form of a gene for a miniV RNA polymerase of the invention, the gene can be expressed in a host cell which expresses the T7 RNA polymerase, or the corresponding T7-like RNA polymerase for the promoter used, wherein the RNA polymerase for the promoter is expressed either constitutively, or more preferably, from an inducible promoter. By way of example, a T7 RNA polymerase expression system, such as, but not limited to, the expression systems disclosed in, for example, U.S. Patent Nos. 5,693,489 and 5,869,320, the disclosures of which are incorporated herein by reference in their entirety.

***b. Enhancers***

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like

promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB, http://www.epd.isb-sib.ch/) could also be used to drive expression. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

**Table 3 – Promoter and Enhancer Elements**

| Promoter/Enhancer | References |
|---|---|
| Immunoglobulin Heavy Chain | Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990 |
| Immunoglobulin Light Chain | Queen and Baltimore, 1983; Picard and Schaffner, 1984 |
| T-Cell Receptor | Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*; 1990 |
| HLA DQ a and DQ β | Sullivan and Peterlin, 1987 |
| β-Interferon | Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988 |
| Interleukin-2 | Greene *et al.*, 1989 |
| Interleukin-2 Receptor | Greene *et al.*, 1989; Lin *et al.*, 1990 |
| MHC Class II 5 | Koch *et al.*, 1989 |
| MHC Class II HLA-Dra | Sherman *et al.*, 1989 |

**Table 3 – Promoter and Enhancer Elements**

| Promoter/Enhancer | References |
|---|---|
| β-Actin | Kawamoto *et al.*, 1988; Ng *et al.*; 1989 |
| Muscle Creatine Kinase | Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989 |
| Prealbumin (Transthyretin) | Costa *et al.*, 1988 |
| Elastase I | Ornitz *et al.*, 1987 |
| Metallothionein | Karin *et al.*, 1987; Culotta and Hamer, 1989 |
| Collagenase | Pinkert *et al.*, 1987; Angel *et al.*, 1987 |
| Albumin Gene | Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990 |
| α-Fetoprotein | Godbout *et al.*, 1988; Campere and Tilghman, 1989 |
| t-Globin | Bodine and Ley, 1987; Perez-Stable and Constantini, 1990 |
| β-Globin | Trudel and Constantini, 1987 |
| e-fos | |
| c-HA-ras | Deschamps *et al.*, 1985 |
| Insulin | Edlund *et al.*, 1985 |
| Neural Cell Adhesion Molecule (NCAM) | Hirsh *et al.*, 1990 |
| $\alpha_{1\text{-Antitrypsin}}$ | Latimer *et al.*, 1990 |
| H2B (TH2B) Histone | Hwang *et al.*, 1990 |
| Mouse or Type I Collagen | Ripe *et al.*, 1989 |
| Glucose-Regulated Proteins (GRP94 and GRP78) | Chang *et al.*, 1989 |
| Rat Growth Hormone | Larsen *et al.*, 1986 |
| Human Serum Amyloid A (SAA) | Edbrooke *et al.*, 1989 |
| Troponin I (TN I) | Yutzey *et al.*, 1989 |
| Platelet-Derived Growth Factor | Pech *et al.*, 1989 |
| Duchenne Muscular Dystrophy | Klamut *et al.*, 1990 |

| Table 3 – Promoter and Enhancer Elements | |
|---|---|
| Promoter/Enhancer | References |
| SV40 | Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988 |
| Polyoma | Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988 |
| Retroviruses | Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Choi *et al.*, 1988; Reisman and Rotter, 1989 |
| Papilloma Virus | Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens and Hentschel, 1987 |
| Hepatitis B Virus | Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988 |
| Human Immunodeficiency Virus | Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989 |
| Cytomegalovirus | Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986 |
| Gibbon Ape Leukemia Virus | Holbrook *et al.*, 1987; Quinn *et al.*, 1989 |

**Table 4 – Inducible Elements**

| Element | Inducer | References |
|---|---|---|
| MT II | Phorbol Ester (TFA) Heavy metals | Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989 |
| MMTV (mouse mammary tumor virus) | Glucocorticoids | Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.* 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988 |
| β-Interferon | Poly(rI)x and Poly(rc) | Tavernier *et al.*, 1983 |
| Adenovirus 5 E2 | Ela | Imperiale and Nevins, 1984 |
| Collagenase | Phorbol Ester (TPA) | Angel *et al.*, 1987a |
| Stromelysin | Phorbol Ester (TPA) | Angel *et al.*, 1987b |
| SV40 | Phorbol Ester (TPA) | Angel *et al.*, 1987b |
| Murine MX Gene | Interferon, Newcastle Disease Virus | |
| GRP78 Gene | A23187 | Resendez *et al.*, 1988 |
| α-2-Macroglobulin | IL-6 | Kunz *et al.*, 1989 |
| Vimentin | Serum | Rittling *et al.*, 1989 |
| MHC Class I Gene H-2κb | Interferon | Blanar *et al.*, 1989 |
| HSP70 | Ela, SV40 Large T Antigen | Taylor *et al.*, 1989; Taylor and Kingston, 1990a, b |
| Proliferin | Phorbol Ester-TPA | Mordacq and Linzer, 1989 |
| Tumor Necrosis Factor | FMA | Hensel *et al.*, 1989 |
| Thyroid Stimulating Hormone a Gene | Thyroid Hormone | Chatterjee *et al.*, 1989 |

Turning to the expression of the proteinaceous molecules after transcription using the vRNAP, mini-vRNAP, or mutants thereof of the present invention, once a suitable clone or

clones have been obtained, whether they be cDNA based or genomic, one may proceed to prepare an expression system. The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. It is believed that virtually any expression system may be employed in the expression of the proteinaceous molecules of the present invention.

Both cDNA and genomic sequences are suitable for eukaryotic expression, as the host cell will generally process the genomic transcripts to yield functional mRNA for translation into proteinaceous molecules. Generally speaking, it may be more convenient to employ as the recombinant gene a cDNA version of the gene. It is believed that the use of a cDNA version will provide advantages in that the size of the gene will generally be much smaller and more readily employed to transfect the targeted cell than will a genomic gene, which will typically be up to an order of magnitude or more larger than the cDNA gene. However, it is contemplated that a genomic version of a particular gene may be employed where desired.

In expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

### *c. Antisense and Ribozymes*

In some embodiments of the invention the vRNA polymerase can be used to synthesize antisense RNA or ribozymes.

The term "antisense nucleic acid" is intended to refer to the oligonucleotides complementary to the base sequences of DNA and RNA. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target nucleic acid and interfere with transcription, RNA processing, transport, translation, and/or stability. Targeting double-stranded (ds) DNA with oligonucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. An antisense nucleic acid may be complementary to SEQ ID NO:1, 3, 5, 7 or 14, complementary to a mini-vRNAP encoding sequence or to mini-vRNAP non-coding

sequences. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries (splice junctions) of a gene. It is contemplated that the most effective antisense constructs may include regions complementary to intron/exon splice junctions. Thus, antisense constructs with complementary regions within 50-200 bases of an intron-exon splice junction may be used. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, "complementary" or "antisense" means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence 17 bases long should occur only once in the human

genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs will be used. One can readily determine whether a given antisense nucleic acid is effective at targeting of the corresponding host cell gene simply by testing the constructs *in vivo* to determine whether the endogenous gene's function is affected or whether the expression of related genes having complementary sequences is affected.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1993).

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in oncogene DNA and RNA. Ribozymes either can be targeted directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as an expression construct encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense nucleic acids. Sequences for ribozymes may be included in the DNA template to eliminate undesired 5' end sequences in RNAs generated through T7 RNA polymerase transcription.

Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlack *et al.*, 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U.S. Patent 5,354,855 reports that certain ribozymes can act as endonucleases with a sequence specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990; Sioud *et al.*, 1992). Recently, it was reported that ribozymes elicited genetic changes in some cell lines to which they were applied; the altered genes included the oncogenes H-*ras*, c-*fos* and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme. In light of the information included herein and the knowledge of one of ordinary skill in the art, the preparation and use of additional ribozymes that are specifically targeted to a given gene will now be straightforward.

Several different ribozyme motifs have been described with RNA cleavage activity (reviewed in Symons, 1992). Examples of ribozymes include sequences from the Group I self-splicing introns including tobacco ringspot virus (Prody, *et al.*, 1986), avocado sunblotch viroid (Palukaitis, *et al.*, 1979; Symons, 1981), and Lucerne transient streak virus (Forster and Symons, 1987). Sequences from these and related viruses are referred to as hammerhead ribozymes based on a predicted folded secondary structure.

Other suitable ribozymes include sequences from RNase P with RNA cleavage activity (Yuan, *et al.*, 1992; Yuan and Altman, 1994), hairpin ribozyme structures (Berzal-Herranz, *et al.*, 1992; Chowrira *et al.*, 1993) and hepatitis δ virus based ribozymes (Perrotta and Been, 1992). The general design and optimization of ribozyme directed RNA cleavage activity has been discussed in detail (Haseloff and Gerlach, 1988; Symons, 1992; Chowrira, *et al.*, 1994; and Thompson, *et al.*, 1995).

The other variable on ribozyme design is the selection of a cleavage site on a given target RNA. Ribozymes are targeted to a given sequence by virtue of annealing to a site by complementary base pair interactions. Two stretches of homology are required for this targeting. These stretches of homologous sequences flank the catalytic ribozyme structure defined above. Each stretch of homologous sequence can vary in length from 7 to 15 nucleotides. The only requirement for defining the homologous sequences is that, on the target RNA, they are separated by a specific sequence which is the cleavage site. For hammerhead ribozymes, the

cleavage site is a dinucleotide sequence on the target RNA, uracil (U) followed by either an adenine, cytosine or uracil (A, C or U; Perriman, *et al.*, 1992; Thompson, *et al.*, 1995). The frequency of this dinucleotide occurring in any given RNA is statistically 3 out of 16. Therefore, for a given target messenger RNA of 1000 bases, 187 dinucleotide cleavage sites are statistically possible.

Designing and testing ribozymes for efficient cleavage of a target RNA is a process well known to those skilled in the art. Examples of scientific methods for designing and testing ribozymes are described by Chowrira *et al.* (1994) and Lieber and Strauss (1995), each incorporated by reference. The identification of operative and preferred sequences for use in ribozymes is simply a matter of preparing and testing a given sequence, and is a routinely practiced "screening" method known to those of skill in the art.

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

### *d. Host Cells*

Host cells may be derived from prokaryotes or eukaryotes, including yeast cells, insect cells, and mammalian cells, depending upon whether the desired result is replication of the vector or expression of part or all of the vector-encoded nucleic acid sequences. Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials (www.atcc.org). An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryotic host cell for replication of many vector copies. Bacterial cells used as host cells for vector replication and/or expression include DH5α, BL 21, JM109, and KC8, as well as a number of commercially available bacterial hosts

such as SURE® Competent Cells and SOLOPACK Gold Cells (STRATAGENE®, La Jolla, CA). Alternatively, bacterial cells such as *E. coli* LE392 could be used as host cells. Appropriate yeast cells include *Saccharomyces cerevisiae*, *Saccharomyces pombe*, and *Pichia pastoris*.

Examples of eukaryotic host cells for replication and/or expression of a vector include HeLa, NIH3T3, Jurrat, 293, Cos, CHO, Saos, BHK, C127 and PC12. Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with either a eukaryotic or prokaryotic host cell, particularly one that is permissive for replication or expression of the vector.

Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and/or their cognate polypeptides, proteins, or peptides.

It is proposed that vRNAP, or more particularly mini-vRNAP may be co-expressed with other selected proteinaceous molecules such as *Eco*SSB and other proteins of interest, wherein the proteinaceous molecules may be co-expressed in the same cell or vRNAP gene may be provided to a cell that already has another selected proteinaceous molecule. Co-expression may be achieved by co-transfecting the cell with two distinct recombinant vectors, each bearing a copy of either of the respective DNAs. Alternatively, a single recombinant vector may be constructed to include the coding regions for both of the proteinaceous molecules, which could then be expressed in cells transfected with the single vector. In either event, the term "co-expression" herein refers to the expression of both the vRNAP gene and the other selected proteinaceous molecules in the same recombinant cell.

As used herein, the terms "engineered" and "recombinant" cells or host cells are intended to refer to a cell into which an exogenous DNA segment or gene, such as a cDNA or gene encoding vRNAP, mini-vRNAP or a mutant thereof, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced exogenous DNA segment or gene. Engineered cells are thus cells having a gene or genes introduced through the hand of man. Recombinant cells include those having an

introduced cDNA or genomic gene, and also include genes positioned adjacent to a promoter not naturally associated with the particular introduced gene.

To express a recombinant vRNAP, whether mutant or wild-type, in accordance with the present invention one would prepare an expression vector that comprises a wild-type, or mutant vRNAP proteinaceous molecule-encoding nucleic acid under the control of one or more promoters. To bring a coding sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter directs transcription of the DNA and promotes expression of the encoded recombinant protein, polypeptide or peptide. This is the meaning of "recombinant expression" in this context.

Many standard techniques are available to construct expression vectors containing the appropriate nucleic acids and transcriptional/translational control sequences in order to achieve protein, polypeptide or peptide expression in a variety of host expression systems. Cell types available for expression include, but are not limited to, bacteria, such as *E. coli* and *B. subtilis,* transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors.

Certain examples of prokaryotic hosts are *E. coli* strain RR1, *E. coli* LE392, *E. coli* B, *E. coli* X 1776 (ATCC No. 31537) as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325); bacilli such as *Bacillus subtilis*; and other enterobacteriaceae such as *Salmonella typhimurium*, *Serratia marcescens*, and various *Pseudomonas* species.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication origin, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is often transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, the phage lambda GEM$^{TM}$-11 may be utilized in making a recombinant phage vector which can be used to transform host cells, such as *E. coli* LE392.

Further useful vectors include pIN vectors (Inouye *et al.*, 1985); and pGEX vectors, for use in generating glutathione S-transferase (GST) soluble proteins for later purification and separation or cleavage.

The following details concerning recombinant protein production in bacterial cells, such as *E. coli*, are provided by way of exemplary information on recombinant protein production in general, the adaptation of which to a particular recombinant expression system will be known to those of skill in the art.

Bacterial cells, for example, *E. coli*, containing the expression vector are grown in any of a number of suitable media, for example, LB. The expression of the recombinant proteinaceous molecule may be induced, *e.g.*, by adding IPTG or any appropriate inducer to the media or by switching incubation to a higher temperature, depending on the regulated promoter used. After culturing the bacteria for a further period, generally of between 2 and 24 hours, the cells are collected by centrifugation and washed to remove residual media.

The bacterial cells are then lysed, for example, by disruption in a cell homogenizer, by sonication or cell press and centrifuged to separate the dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby the dense inclusion bodies are selectively enriched by incorporation of sugars, such as sucrose, into the buffer and centrifugation at a selective speed.

If the recombinant proteinaceous molecule is expressed in the inclusion bodies, as is the case in many instances, these can be washed in any of several solutions to remove some of the contaminating host proteins, then solubilized in solutions containing high concentrations of urea (*e.g.*, 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents, such as β-mercaptoethanol or DTT (dithiothreitol).

Under some circumstances, it may be advantageous to incubate the proteinaceous molecule for several hours under conditions suitable for the proteinaceous molecule to undergo a refolding process into a conformation which more closely resembles that of the native proteinaceous molecule. Such conditions generally include low proteinaceous molecule concentrations, less than 500 mg/ml, low levels of reducing agent, concentrations of urea less than 2 M and often the presence of reagents such as a mixture of reduced and oxidized glutathione which facilitate the interchange of disulfide bonds within the proteinaceous molecule.

The refolding process can be monitored, for example, by SDS-PAGE, or with antibodies specific for the native molecule (which can be obtained from animals vaccinated with the native molecule or smaller quantities of recombinant proteinaceous molecule). Following refolding, the proteinaceous molecule can then be purified further and separated from the refolding mixture by chromatography on any of several supports including ion exchange resins, gel permeation resins or on a variety of affinity columns.

For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used. This plasmid already contains the *trp*l gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1. The presence of the *trp*l lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoter sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate protein, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

In addition to micro-organisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. In addition to mammalian cells, these include insect cell

systems infected with recombinant virus expression vectors (*e.g.*, baculovirus); and plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing one or more RNAP coding sequences.

Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteinaceous molecules. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign proteinaceous molecule expressed.

A number of viral-based expression systems may be utilized, for example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *Hind*III site toward the *BglI* site located in the viral origin of replication.

In cases where an adenovirus is used as an expression vector, the coding sequences may be ligated to an adenovirus transcription/ translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1, E3, or E4) will result in a recombinant virus that is viable and capable of expressing an RNA in infected hosts.

Specific initiation signals may also be used for more efficient translation using the vRNAP of the current invention. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may additionally need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be in-frame (or in-phase) with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression

may be enhanced by the inclusion of appropriate transcription enhancer elements and transcription terminators.

In eukaryotic expression, one will also typically desire to incorporate into the transcriptional unit an appropriate polyadenylation site (*e.g.*, 5'-AATAAA-3') if one was not contained within the original cloned segment. Typically, the poly A addition site is placed about 30 to 2000 nucleotides "downstream" of the termination site of the proteinaceous molecule at a position prior to transcription termination.

For long-term, high-yield production of a recombinant vRNAP protein, polypeptide or peptide, stable expression is preferred. For example, cell lines that stably express constructs encoding a vRNAP protein, polypeptide or peptide may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with vectors controlled by appropriate expression control elements (*e.g.*, promoter, enhancer sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

A number of selection systems may be used, including, but not limited to, the herpes simplex virus thymidine kinase (tk), hypoxanthine-guanine phosphoribosyltransferase (hgprt) and adenine phosphoribosyltransferase (aprt) genes, in $tk^-$, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (dhfr), that confers resistance to methotrexate; gpt, that confers resistance to mycophenolic acid; neomycin (neo), that confers resistance to the aminoglycoside G-418; and hygromycin (hygro), that confers resistance to hygromycin.

Large scale suspension culture of bacterial cells in stirred tanks is a common method for production of recombinant proteinaceous molecules. Two suspension culture reactor designs are in wide use -- the stirred reactor and the airlift reactor. The stirred design has successfully been used on an 8000 liter capacity for the production of interferon. Cells are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The culture is usually mixed with one or

more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

The airlift reactor for microbial fermentation relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easily, has good mass transfer of gases and generates relatively low shear forces.

It is contemplated that the vRNAP proteins, polypeptides or peptides of the invention may be "overexpressed," *i.e.*, expressed in increased levels relative to its natural expression in cells. Such overexpression may be assessed by a variety of methods, including radio-labeling and/or proteinaceous molecule purification. However, simple and direct methods are preferred, for example, those involving SDS/PAGE and proteinaceous composition staining or western blotting, followed by quantitative analyses, such as densitometric scanning of the resultant gel or blot. A specific increase in the level of the recombinant protein, polypeptide or peptide in comparison to the level in natural cells is indicative of overexpression, as is a relative abundance of the specific proteinaceous molecule in relation to the other proteins produced by the host cell and, *e.g.*, visible on a gel.

## IV. Methods of Gene Transfer

In order to mediate the effect of transgene expression in a cell, it will be necessary to transfer the expression constructs (*e.g.*, a therapeutic construct) of the present invention into a cell. Such transfer may employ viral or non-viral methods of gene transfer. This section provides a discussion of methods and compositions of gene or nucleic acid transfer, including transfer of antisense sequences.

The vRNAP genes are incorporated into a viral vector to mediate gene transfer to a cell. Additional expression constructs encoding *Eco*SSB and other therapeutic agents as described herein may also be transferred *via* viral transduction using infectious viral particles, for example,

by transformation with an adenovirus vector of the present invention. Alternatively, a retrovirus, bovine papilloma virus, an adeno-associated virus (AAV), a lentiviral vector, a vaccinia virus, a polyoma virus, or an infective virus that has been engineered to express a specific binding ligand may be used. Similarly, nonviral methods which include, but are not limited to, direct delivery of DNA such as by injection, electroporation, calcium phosphate precipitation, liposome mediated transfection, and microprojectile bombardment may be employed. Thus, in one example, viral infection of cells is used in order to deliver therapeutically significant genes to a cell. Typically, the virus simply will be exposed to the appropriate host cell under physiologic conditions, permitting uptake of the virus.

Microinjection can be used for delivery into a cell. Microinjection involves the insertion of a substance such as RNA into a cell through a microelectrode. Typical applications include the injection of drugs, histochemical markers (such as horseradish peroxidase or lucifer yellow) and RNA or DNA in molecular biological studies. To extrude the substances through the very fine electrode tips, either hydrostatic pressure (pressure injection) or electric currents (ionophoresis) is employed.

## V. Proteinaceous Compositions

In certain embodiments, the present invention concerns novel compositions or methods comprising at least one proteinaceous molecule. The proteinaceous molecule may have a sequence essentially as set forth in SEQ ID NO:2, 4, 6, 8 or 15. The proteinaceous molecule may be a vRNAP or more preferably a mini-vRNAP, or a delivery agent. The proteinaceous molecule may also be a mutated mini-vRNAP.

As used herein, a "proteinaceous molecule," "proteinaceous composition," "proteinaceous compound," "proteinaceous chain" or "proteinaceous material" generally refers to, but is not limited to, a protein of greater than about 200 amino acids or the full length endogenous sequence translated from a gene; a polypeptide of greater than about 100 amino acids; and/or a peptide of from about 3 to about 100 amino acids. All the "proteinaceous" terms described above may be used interchangeably herein.

In certain embodiments the size of the at least one proteinaceous molecule may comprise, but is not limited to, about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about

19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 275, about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 475, about 500, about 525, about 550, about 575, about 600, about 625, about 650, about 675, about 700, about 725, about 750, about 775, about 800, about 825, about 850, about 875, about 900, about 925, about 950, about 975, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1750, about 2000, about 2250, about 2500 or greater amino molecule residues, and any range derivable therein.

As used herein, an "amino molecule" refers to any amino acid, amino acid derivative or amino acid mimic as would be known to one of ordinary skill in the art. In certain embodiments, the residues of the proteinaceous molecule are sequential, without any non-amino molecule interrupting the sequence of amino molecule residues. In other embodiments, the sequence may comprise one or more non-amino molecule moieties. In particular embodiments, the sequence of residues of the proteinaceous molecule may be interrupted by one or more non-amino molecule moieties.

Accordingly, the term "proteinaceous composition" encompasses amino molecule sequences comprising at least one of the 20 common amino acids in naturally synthesized proteins, or at least one modified or unusual amino acid, including but not limited to those shown on Table 5 below.

**TABLE 5**

**Modified and Unusual Amino Acids**

| Abbr. | Amino Acid | Abbr. | Amino Acid |
|---|---|---|---|
| Aad | 2-Aminoadipic acid | EtAsn | N-Ethylasparagine |
| Baad | 3- Aminoadipic acid | Hyl | Hydroxylysine |
| Bala | β-alanine, β-Amino-propionic acid | AHyl | allo-Hydroxylysine |
| Abu | 2-Aminobutyric acid | 3Hyp | 3-Hydroxyproline |
| 4Abu | 4- Aminobutyric acid, piperidinic acid | 4Hyp | 4-Hydroxyproline |
| Acp | 6-Aminocaproic acid | Ide | Isodesmosine |
| Ahe | 2-Aminoheptanoic acid | AIle | allo-Isoleucine |
| Aib | 2-Aminoisobutyric acid | MeGly | N-Methylglycine, sarcosine |
| Baib | 3-Aminoisobutyric acid | MeIle | N-Methylisoleucine |
| Apm | 2-Aminopimelic acid | MeLys | 6-N-Methyllysine |
| Dbu | 2,4-Diaminobutyric acid | MeVal | N-Methylvaline |
| Des | Desmosine | Nva | Norvaline |
| Dpm | 2,2'-Diaminopimelic acid | Nle | Norleucine |
| Dpr | 2,3-Diaminopropionic acid | Orn | Ornithine |
| EtGly | N-Ethylglycine | | |

In certain embodiments the proteinaceous composition comprises at least one protein, polypeptide or peptide, such as vRNAP or mini-vRNAP. In further embodiments the proteinaceous composition comprises a biocompatible protein, polypeptide or peptide. As used herein, the term "biocompatible" refers to a substance which produces no significant untoward effects when applied to, or administered to, a given organism according to the methods and amounts described herein. Such untoward or undesirable effects are those such as significant toxicity or adverse immunological reactions. In preferred embodiments, biocompatible protein, polypeptide or peptide containing compositions will generally be mammalian proteins or peptides or synthetic proteins or peptides each essentially free from toxins, pathogens and harmful immunogens.

Proteinaceous compositions may be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, the isolation of proteinaceous compounds from natural sources, or the chemical synthesis of proteinaceous materials. The nucleotide and protein, polypeptide and peptide sequences for various genes have been previously disclosed, and may be found at computerized databases known to those of ordinary skill in the art. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases (http://www.ncbi.nlm.nih.gov/). The coding regions for these known genes may be amplified and/or expressed using the techniques disclosed herein or as would be know to those of ordinary skill in the art. Alternatively, various commercial preparations of proteins, polypeptides and peptides are known to those of skill in the art.

In certain embodiments, a proteinaceous compound may be purified. Generally, "purified" will refer to a specific or desired protein, polypeptide, or peptide composition that has been subjected to fractionation to remove various other proteins, polypeptides, or peptides, and which composition substantially retains its activity, as may be assessed, for example, by the protein assays, as would be known to one of ordinary skill in the art for the specific or desired protein, polypeptide or peptide.

In certain embodiments, the proteinaceous composition may comprise at least one antibody. A mini-vRNAP antibody may comprise all or part of an antibody that specifically recognizes mini-vRNAP. As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting.

The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, $F(ab')_2$, single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

It is contemplated that virtually any protein, polypeptide or peptide containing component may be used in the compositions and methods disclosed herein. However, it is preferred that the proteinaceous material is biocompatible. In certain embodiments, it is envisioned that the formation of a more viscous composition will be advantageous in that the high viscosity will allow the composition to be more precisely or easily applied to the tissue and to be maintained in contact with the tissue throughout the procedure. In such cases, the use of a peptide composition, or more preferably, a polypeptide or protein composition, is contemplated. Ranges of viscosity include, but are not limited to, about 40 to about 100 poise. In certain aspects, a viscosity of about 80 to about 100 poise is preferred.

Proteins and peptides suitable for use in this invention may be autologous proteins or peptides, although the invention is clearly not limited to the use of such autologous proteins. As used herein, the term "autologous protein, polypeptide or peptide" refers to a protein, polypeptide or peptide which is derived or obtained from an organism. Organisms that may be used include, but are not limited to, a bovine, a reptilian, an amphibian, a piscine, a rodent, an avian, a canine, a feline, a fungal, a plant, or a prokaryotic organism, with a selected animal or human subject being preferred. The "autologous protein, polypeptide or peptide" may then be used as a component of a composition intended for application to the selected animal or human subject. In certain aspects, the autologous proteins or peptides are prepared, for example from whole plasma of the selected donor. The plasma is placed in tubes and placed in a freezer at about -80°C for at least about 12 hours and then centrifuged at about 12,000 times g for about 15 minutes to obtain the precipitate. The precipitate, such as fibrinogen may be stored for up to about one year (Oz, 1990).

## VI. Protein Purification

To prepare a composition comprising a vRNAP or mini-vRNAP, it is desirable to purify the components or variants thereof. Purification of the mini-vRNAP (SEQ ID NO:4) can be done in two step using affinity columns. The mini-vRNAP of SEQ ID NO:6 has been modified to comprise a His tag such that purification can be done in a single step when using metal affinity columns such as those which employ nickel, cobalt or zinc. The full length vRNAP of SEQ ID NO:15 is also His tagged for purification.

According to one embodiment of the present invention, purification of a peptide comprising vRNAP can be utilized ultimately to operatively link this domain with a selective

agent. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is affinity chromatography.

A tag may be used for protein or peptide purification and detection such as hexahistidine (6-His, HHHHHH), FLAG (DYKDDDDK), hemaglutinin (HA, YPYDVPDYA) and c-myc (EQKLISEEDL). Other tags also have been generated, most of which are very small, comprising only a few amino acids, and are therefore likely to have little to no effect on the conformation of the mature protein or peptide. These small tags do not require any special conformation to be recognized by antibodies. Systems for protein purification using these tags include NTA resin (6-His) or the FLAG fusion system marketed by IBI (FLAG) where the fusion protein is affinity-purified on an antibody column.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide, such as a vRNAP. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a protein or peptide composition, such as the vRNAP, that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for

example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a "-fold purification" number. The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

Ion exchange chromatography is a preferred method of separation. Using columns resins such as the metal affinity chromatography resin TALON are also preferred. TALON resin has

an enhanced resolving power for polyhistidine-tagged proteins. This results in greater purity with less effort. TALON employs cobalt, an electropositive metal with a remarkably high affinity for polyhistidine-tagged proteins and a low affinity for other proteins. Often, no discernible binding of host proteins occurs and a separate wash step is not required. The binding properties of cobalt allow protein elution under mild pH conditions that protect protein integrity.

Further concentration of the proteins can be done on an anion exchange column, such as the MonoQ column, a high resolution, anion exchange column. This column works at pressures less than 5 MPa, has a high capacity and gives very high chromatographic resolution.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity chromatography, a particularly efficient method of purifying peptides, is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble

matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (*e.g.*, alter pH, ionic strength, and temperature). Tags, as described herein above, can be used in affinity chromatography.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand also should provide relatively tight binding, and it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accordance with the present invention is discussed below.

An affinity column may have an N4 promoter which the vRNAP or mini-vRNAP proteins recognize attached to a matrix. This column would be suitable for use for the purification of polymerases with no additional tags such as histidine tags.

## VII. Separation, Quantitation, and Identification Methods

Following synthesis of the RNA, it may be desirable to separate the amplification products of several different lengths from each other and from the template and the excess primer.

### *a. Gel Electrophoresis*

In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989).

### *b. Chromatographic Techniques*

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography (Freifelder, 1982). In yet another alternative, labeled cDNA products, such as biotin-labeled or antigen-labeled, can be captured with beads bearing avidin or antibody, respectively.

### *c. Microfluidic Techniques*

Microfluidic techniques include separation on a platform such as microcapillaries, designed by ACLARA BioSciences Inc., or the LabChip™ "liquid integrated circuits" made by Caliper Technologies Inc. These microfluidic platforms require only nanoliter volumes of sample, in contrast to the microliter volumes required by other separation technologies. Miniaturizing some of the processes involved in genetic analysis has been achieved using microfluidic devices. For example, published PCT Application No. WO 94/05414, to Northrup and White, incorporated herein by reference, reports an integrated micro-PCR™ apparatus for collection and amplification of nucleic acids from a specimen. U.S. Patent Nos. 5,304,487 to Wilding *et al.*, and 5,296,375 to Kricka *et al.*, discuss devices for collection and analysis of cell containing samples and are incorporated herein by reference. U.S. Patent No. 5,856,174 describes an apparatus which combines the various processing and analytical operations involved in nucleic acid analysis and is incorporated herein by reference.

### *d. Capillary Electrophoresis*

In some embodiments, it may be desirable to provide an additional, or alternative means for analyzing the amplified genes. In these embodiments, micro capillary arrays are contemplated to be used for the analysis.

Microcapillary array electrophoresis generally involves the use of a thin capillary or channel which may or may not be filled with a particular separation medium. Electrophoresis of a sample through the capillary provides a size based separation profile for the sample. The use of microcapillary electrophoresis in size separation of nucleic acids has been reported in, *e.g.*, Woolley and Mathies, 1994. Microcapillary array electrophoresis generally provides a rapid method for size-based sequencing, PCR™ product analysis and restriction fragment sizing. The high surface to volume ratio of these capillaries allows for the application of higher electric fields across the capillary without substantial thermal variation across the capillary, consequently allowing for more rapid separations. Furthermore, when combined with confocal imaging methods, these methods provide sensitivity in the range of attomoles, which is comparable to the sensitivity of radioactive sequencing methods. Microfabrication of microfluidic devices including microcapillary electrophoretic devices has been discussed in detail in, *e.g.*, Jacobsen *et al.*, 1994; Effenhauser *et al.*, 1994; Harrison *et al.*, 1993; Effenhauser *et al.*, 1993; Manz *et al.*, 1992; and U.S. Patent No. 5,904,824. Typically, these methods comprise photolithographic etching of micron scale channels on a silica, silicon or other crystalline substrate or chip, and can

be readily adapted for use in the present invention. In some embodiments, the capillary arrays may be fabricated from the same polymeric materials described for the fabrication of the body of the device, using the injection molding techniques described herein.

Tsuda *et al.,* 1990, describes rectangular capillaries, an alternative to the cylindrical capillary glass tubes. Some advantages of these systems are their efficient heat dissipation due to the large height-to-width ratio and, hence, their high surface-to-volume ratio and their high detection sensitivity for optical on-column detection modes. These flat separation channels have the ability to perform two-dimensional separations, with one force being applied across the separation channel, and with the sample zones detected by the use of a multi-channel array detector.

In many capillary electrophoresis methods, the capillaries, *e.g.*, fused silica capillaries or channels etched, machined or molded into planar substrates, are filled with an appropriate separation/sieving matrix. Typically, a variety of sieving matrices are known in the art may be used in the microcapillary arrays. Examples of such matrices include, *e.g.*, hydroxyethyl cellulose, polyacrylamide, agarose and the like. Generally, the specific gel matrix, running buffers and running conditions are selected to maximize the separation characteristics of the particular application, *e.g.*, the size of the nucleic acid fragments, the required resolution, and the presence of native or undenatured nucleic acid molecules. For example, running buffers may include denaturants, chaotropic agents such as urea or the like, to denature nucleic acids in the sample.

*e. Mass Spectroscopy*

Mass spectrometry provides a means of "weighing" individual molecules by ionizing the molecules *in vacuo* and making them "fly" by volatilization. Under the influence of combinations of electric and magnetic fields, the ions follow trajectories depending on their individual mass (m) and charge (z). For low molecular weight molecules, mass spectrometry has been part of the routine physical-organic repertoire for analysis and characterization of organic molecules by the determination of the mass of the parent molecular ion. In addition, by arranging collisions of this parent molecular ion with other particles (*e.g.*, argon atoms), the molecular ion is fragmented forming secondary ions by the so-called collision induced dissociation (CID). The fragmentation pattern/pathway very often allows the derivation of detailed structural information. Other applications of mass spectrometric methods known in the art can be found summarized in

Methods in Enzymology, Vol. 193: "Mass Spectrometry" (J. A. McCloskey, editor), 1990, Academic Press, New York.

Due to the apparent analytical advantages of mass spectrometry in providing high detection sensitivity, accuracy of mass measurements, detailed structural information by CID in conjunction with an MS/MS configuration and speed, as well as on-line data transfer to a computer, there has been considerable interest in the use of mass spectrometry for the structural analysis of nucleic acids. Reviews summarizing this field include K. H. Schram (1990); and P. F. Crain (1990). The biggest hurdle to applying mass spectrometry to nucleic acids is the difficulty of volatilizing these very polar biopolymers. Therefore, "sequencing" had been limited to low molecular weight synthetic oligonucleotides by determining the mass of the parent molecular ion and through this, confirming the already known sequence, or alternatively, confirming the known sequence through the generation of secondary ions (fragment ions) via CID in an MS/MS configuration utilizing, in particular, for the ionization and volatilization, the method of fast atomic bombardment (FAB mass spectrometry) or plasma desorption (PD mass spectrometry). As an example, the application of FAB to the analysis of protected dimeric blocks for chemical synthesis of oligodeoxynucleotides has been described (Koster *et al.* 1987).

Two ionization/desorption techniques are electrospray/ionspray (ES) and matrix-assisted laser desorption/ionization (MALDI). ES mass spectrometry was introduced by Fenn *et al.* 1984; WO 90/14148 and its applications are summarized in review articles (R. D. Smith *et al.* 1990; B. Ardrey, 1992). As a mass analyzer, a quadrupole is most frequently used. The determination of molecular weights in femtomole amounts of sample is very accurate due to the presence of multiple ion peaks, which all could be used for the mass calculation.

MALDI mass spectrometry, in contrast, can be particularly attractive when a time-of-flight (TOF) configuration is used as a mass analyzer. The MALDI-TOF mass spectrometry has been introduced by Hillenkamp *et al.* (1990). Since, in most cases, no multiple molecular ion peaks are produced with this technique, the mass spectra, in principle, look simpler compared to ES mass spectrometry. DNA molecules up to a molecular weight of 410,000 Daltons could be desorbed and volatilized (Williams *et al.*, 1989). More recently, the use of infra red lasers (IR) in this technique (as opposed to UV-lasers) has been shown to provide mass spectra of larger nucleic acids such as synthetic DNA, restriction enzyme fragments of plasmid DNA, and RNA transcripts up to a size of 2180 nucleotides (Berkenkamp *et al.*, 1998). Berkenkamp *et al.*, 1998, also

describe how DNA and RNA samples can be analyzed by limited sample purification using MALDI-TOF IR.

In Japanese Patent No. 59-131909, an instrument is described which detects nucleic acid fragments separated either by electrophoresis, liquid chromatography or high speed gel filtration. Mass spectrometric detection is achieved by incorporating into the nucleic acids atoms which normally do not occur in DNA such as S, Br, I or Ag, Au, Pt, Os, Hg.

### *f. Energy Transfer*

Labeling hybridization oligonucleotide probes with fluorescent labels is a well known technique in the art and is a sensitive, nonradioactive method for facilitating detection of probe hybridization. More recently developed detection methods employ the process of fluorescence energy transfer (FET) rather than direct detection of fluorescence intensity for detection of probe hybridization. FET occurs between a donor fluorophore and an acceptor dye (which may or may not be a fluorophore) when the absorption spectrum of one (the acceptor) overlaps the emission spectrum of the other (the donor) and the two dyes are in close proximity. Dyes with these properties are referred to as donor/acceptor dye pairs or energy transfer dye pairs. The excited-state energy of the donor fluorophore is transferred by a resonance dipole-induced dipole interaction to the neighboring acceptor. This results in quenching of donor fluorescence. In some cases, if the acceptor is also a fluorophore, the intensity of its fluorescence may be enhanced. The efficiency of energy transfer is highly dependent on the distance between the donor and acceptor, and equations predicting these relationships have been developed (Forster, 1948). The distance between donor and acceptor dyes at which energy transfer efficiency is 50% is referred to as the Forster distance ($R_O$). Other mechanisms of fluorescence quenching are also known including, for example, charge transfer and collisional quenching.

Energy transfer and other mechanisms which rely on the interaction of two dyes in close proximity to produce quenching are an attractive means for detecting or identifying nucleotide sequences, as such assays may be conducted in homogeneous formats. Homogeneous assay formats are simpler than conventional probe hybridization assays which rely on detection of the fluorescence of a single fluorophore label, as heterogeneous assays generally require additional steps to separate hybridized label from free label. Several formats for FET hybridization assays are reviewed in Nonisotopic DNA Probe Techniques (1992).

Homogeneous methods employing energy transfer or other mechanisms of fluorescence quenching for detection of nucleic acid amplification have also been described. Higuchi *et al.* (1992) disclose methods for detecting DNA amplification in real-time by monitoring increased fluorescence of ethidium bromide as it binds to double-stranded DNA. The sensitivity of this method is limited because binding of the ethidium bromide is not target specific and background amplification products are also detected. Lee, *et al.* (1993) disclose a real-time detection method in which a doubly-labeled detector probe is cleaved in a target amplification-specific manner during PCR™. The detector probe is hybridized downstream of the amplification primer so that the 5′-3′ exonuclease activity of Taq polymerase digests the detector probe, separating two fluorescent dyes which form an energy transfer pair. Fluorescence intensity increases as the probe is cleaved. WO 96/21144 discloses continuous fluorometric assays in which enzyme-mediated cleavage of nucleic acids results in increased fluorescence. Fluorescence energy transfer is suggested for use in the methods, but only in the context of a method employing a single fluorescent label which is quenched by hybridization to the target.

Signal primers or detector probes which hybridize to the target sequence downstream of the hybridization site of the amplification primers have been described for use in detection of nucleic acid amplification (U.S. Pat. No. 5,547,861). The signal primer is extended by the polymerase in a manner similar to extension of the amplification primers. Extension of the amplification primer displaces the extension product of the signal primer in a target amplification-dependent manner, producing a double-stranded secondary amplification product which may be detected as an indication of target amplification. The secondary amplification products generated from signal primers may be detected by means of a variety of labels and reporter groups, restriction sites in the signal primer which are cleaved to produce fragments of a characteristic size, capture groups, and structural features such as triple helices and recognition sites for double-stranded DNA binding proteins.

Many donor/acceptor dye pairs known in the art and may be used in the present invention. These include, for example, fluorescein isothiocyanate (FITC)/tetramethylrhodamine isothiocyanate (TRITC), FITC/Texas Red (Molecular Probes), FITC/N-hydroxysuccinimidyl 1-pyrenebutyrate (PYB), FITC/eosin isothiocyanate (EITC), N-hydroxysuccinimidyl 1-pyrenesulfonate (PYS)/FITC, FITC/Rhodamine X, FITC/tetramethylrhodamine (TAMRA), and others. The selection of a particular donor/acceptor fluorophore pair is not critical. For energy transfer quenching mechanisms, it is only necessary that the emission wavelengths of the donor

fluorophore overlap the excitation wavelengths of the acceptor, *i.e.*, there must be sufficient spectral overlap between the two dyes to allow efficient energy transfer, charge transfer or fluorescence quenching. P-(dimethyl aminophenylazo) benzoic acid (DABCYL) is a non-fluorescent acceptor dye which effectively quenches fluorescence from an adjacent fluorophore, *e.g.*, fluorescein or 5-(2′-aminoethyl) aminonaphthalene (EDANS). Any dye pair which produces fluorescence quenching in the detector nucleic acids of the invention are suitable for use in the methods of the invention, regardless of the mechanism by which quenching occurs. Terminal and internal labeling methods are both known in the art and may be routinely used to link the donor and acceptor dyes at their respective sites in the detector nucleic acid.

### *g. In Vitro Studies*

The synthesized RNA of the current invention may be used for *in vitro* studies of spliceosome assembly, splicing reactions, or antisense experiments.

The spliceosome is a large, multisubunit complex consisting of small, nuclear ribonucleoprotein particles (snRNPs). There are a total of 5 snRNAs: U1, U2, U4, U5, and U6 which are small and uridine rich. Each snRNP has 1 or 2 of these RNAs. In addition to catalyzing the splicing reaction, the spliceosome retains intermediate products, positions splice sites for precise joining of the exons, and prevents exons from diffusing away after cleavage and before ligation. Spliceosome catalysis involves concerted cleavage/ligation reactions in which the 2'-OH of branch site A attacks the 5' splice site to form a 2'-5' phosphodiester bond with the first nucleotide of the intron. The resulting 3'-OH at the end of the 5' exon attacks the 3' splice site to release the lariat form of the intron and join the two exons together with a normal 3'-5' phosphodiester bond. At least 50 different proteins are involved in spliceosome assembly and function. In the group I and group II introns, splicing is improved (in velocity and accuracy) by protein factors (Coetze *et al.*, 1994; Mohr *et al.*, 1994).

## VIII. Kits

Any of the compositions described herein may be comprised in a kit. In a non-limiting example, a vRNAP or more preferably a mini-vRNAP, a derivatized mini-vRNAP, a mutant vRNAP and/or additional agent, may be comprised in a kit. The kits will thus comprise, in suitable container means, a vRNAP, mini-vRNAP, a derivatized mini-vRNAP, a mutant vRNAP and/or an additional agent of the present invention. The inventors envisage other components

that may be included in a kit. These include but are not limited to immunodetection agents such as peroxidase and alkaline phosphatase linked monoclonal and polyclonal antibodies, immunoprecipitation reagents such as protein A- or protein G- linked beads, immune cell purification reagents such as a TALON or monoQ column, cloning reagents for the purpose of manipulating an expression vector, and protein expression reagents including prokaryotic and eukaryotic cells lines for the purpose of protein expression.

The kits may comprise a suitably aliquoted vRNAP, mini-vRNAP, a derivatized mini-vRNAP, a mutant vRNAP and/or additional agent compositions of the present invention, whether labeled or unlabeled, as may be used to prepare a standard curve for a detection assay. The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the vRNAP, lipid, additional agent, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container means.

The kits of the present invention will also typically include a means for containing the vials in close confinement for commercial sale, such as, *e.g.*, injection and/or blow-molded plastic containers into which the desired vials are retained.

As used herein in the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

## IX. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1

### Identification of a transcriptionally active domain of N4 virion RNA polymerase

To determine the minimal domain possessing RNA polymerase activity, controlled proteolysis was performed followed by catalytic (transcriptional) autolabeling (Hartmann, *et al.*, 1988). Upon incubation of RNA polymerase with a benzaldehyde derivative of the initiating nucleotide, the benzaldehyde group forms a Schiff-base with the ε–amino group of lysines located within 12 Å of the nucleotide-binding site. The crosslinking step was performed in the presence of DNA template because it stimulates binding of the initiating nucleotide. The unstable Schiff-base is converted to a stable secondary amine by reduction under mild conditions with sodium borohydride, with concomitant reduction of any non-reacted benzaldehyde derivative. Addition of the next template-directed $\alpha$–$^{32}$P labeled NTP leads to phosphodiester bond formation and catalytic autolabeling of the transcriptionally active polypeptide. Controlled trypsin proteolysis of vRNAP was performed, followed by catalytic autolabeling and analysis on SDS-PAGE (FIG. 3A). Initially, three proteolytic fragments are generated, of which the smaller two are catalytically active. Upon further incubation with trypsin, a single stable, transcriptionally active product approximately 1,100 amino acids in length remains. N-terminal sequencing of the three initial proteolytic fragments (FIG. 3B) indicated that the stable active polypeptide (mini-vRNAP) corresponds to the middle 1/3 of vRNAP, the region containing the three motifs described above (FIG. 2A, SEQ ID NOS: 3 - 4).

### Example 2

### Cloning and purification of N4 mini-vRNAP

The full-size vRNAP and the mini-vRNAP (SEQ ID NOS: 6 and 15) ORFs were cloned under pBAD control with an N-terminal hexahistidine tag (FIG. 4). The mini-vRNAP domain

was cloned into the pBAD B expression plasmid, which was purchased from Invitrogen. Five restriction enzyme sites within pBAD B have been altered; the SnaI site was converted to a HpaI site, and the PflMI and EcoRV sites were destroyed, all by site-directed mutagenesis. The BstBI and HindIII sites were destroyed by enzyme digestion followed by Klenow treatment and re-ligation. FIG. 5 (left) shows the relative amounts of full-length and mini-vRNAP proteins purified on TALON columns from the same volume of *E. coli* BL21 induced cells. Cloned mini-vRNAP is expressed at 100-fold higher levels than cloned full size vRNAP. Further concentration on a MonoQ column reveals that, in contrast to full size vRNAP, mini-vRNAP is stable after induction (FIG. 5, right). At least 10 mg of mini-vRNAP at a 20 mg/ml concentration are obtained from 1 L of induced cells in just two purification steps: TALON and MonoQ minicolumns. A non-histagged version of mini-vRNAP has also been cloned (SEQ ID NO:4). In this case, the enzyme is purified from a crude extract of induced cells in two steps: a promoter DNA-affinity column and MonoQ.

Mini-vRNAP possesses a high binding affinity (Kd =1nM) for N4 promoter-containing DNA oligonucleotides. This property was used for purification of non-his tagged mini-vRNAP (SEQ ID NO:4) on a DNA-affinity column. The column was prepared by adsorbing a 5' biotinylated N4 promoter-containing DNA oligonucleotide onto the matrix of a 1 ml HiTrap Streptavidin column (Pharmacia/Amersham Cat.#17-5112-01) according to the manufacturer's instructions. A debris-free sonicate of bacterial cells expressing mini-vRNAP was passed through the column. To bind mini vRNAP to the DNA-affinity column, the pH in the extract and binding/washing buffer should be between 5 to 9, and the NaCl concentration should be between 50mM and 2M. Nucleases in the extract are inhibited by addition of 2mM EDTA. After washing the column, mini-vRNAP was eluted with warm (25°C) water; the elution temperature was raised from 4°C to 25°C to increase mini-vRNAP recovery. For complete elution, the temperature can be raised up to 43°C without significant change in the quality of the preparation. Elution under these conditions occurs due to the removal of metal ions and consequent melting of the promoter hairpin and dissociation of mini-vRNAP. Different DNA oligonucleotides containing variants of the P2 promoter (SEQ ID NOS: 16 - 19), were used in DNA-affinity columns and tested in mini-vRNAP affinity purification. The best yield was achieved using the DNA oligonucleotide of SEQ ID NO:16. However, the DNA oligonucleotides of SEQ ID NOS: 19 - 20 require a lower temperature than the DNA oligonucleotide of SEQ ID NO:16 for complete elution of the protein, in agreement with the lower thermal stability of the respective promoter hairpins.

Up to 1 mg of mini-vRNAP of 90% purity is obtained from a crude extract of 100 ml *E. coli* culture expressing mini-vRNAP in a single purification step using a 1 ml DNA-affinity column. The binding capacity of the DNA-affinity column was not detectably decreased by multiple use.

## Example 3

## Effect of EcoSSB on transcription of single-stranded templates

Inventors have previously shown that *Eco*SSB is required for N4 vRNAP transcription *in vivo* (Glucksmann, *et al.*, 1992). *Eco*SSB is unique in that, unlike other SSBs whose effect on vRNAP transcription was tested, it does not melt the promoter hairpin structure (Glucksmann-Kuis, *et al.*, 1996). Recently, inventors have reinvestigated the effect of *Eco*SSB on vRNAP transcription of single-stranded templates. FIG. 6 shows transcription in the absence and presence of *Eco* SSB at three different ssDNA template concentrations. The extent of *Eco*SSB activation is template-concentration dependent, with highest activation at low DNA template concentration. These results suggest that *Eco*SSB overcomes template limitation on ssDNA templates.

To further explore this hypothesis, the effect of addition of template or *Eco*SSB to transcription reactions after 20 min incubation in the absence of *Eco*SSB was tested. The transcription reaction mixtures (5-50 μl) contained 20 mM Tris-HCl (pH 7.9 at 25°C), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, 0.01-1 μM mini-vRNAP, 1-100 nM ssDNA template (30-100 nt long, synthesized by Integrated DNA Technologies), 1 mM each of 3 non-labeled NTPs, 0.1 mM $\alpha$-$^{32}$P NTP (1-2 Ci/mmol, NEN), and 1-10 μM *E. coli* SSB. Incubation was for 1 to 80 min at 37°C at the indicated temperature. In the presence of *Eco*SSB, RNA synthesis increased linearly throughout the period of incubation (FIG. 7C). In the absence of *Eco*SSB, no increase in transcription was observed beyond 10 min of incubation (FIG. 7A). Addition of template at 20 min to the reaction carried out in the absence of *Eco*SSB led to a dramatic increase in RNA synthesis (FIG. 7B). Addition of *Eco*SSB at 20 min led to a slow rate of transcriptional recovery (FIG. 7D). These results suggest that *Eco*SSB converts the template from a transcriptionally inactive RNA: DNA hybrid to transcriptionally active single-stranded DNA.

To test this hypothesis, the physical states of the DNA template and the RNA product were analyzed by native gel electrophoresis in the absence and in the presence of *Eco*SSB. In order to have effective transcription in the absence of *Eco*SSB, transcription was performed at an intermediate (5 nM) DNA concentration, at which only a 2-fold effect of *Eco*SSB is observed.

The results of this experiment are shown in FIG. 8. Either $^{32}$P-labeled template (right panel) or labeled NTPs (left panel) were used to analyze the state of the template (right panel) or RNA product (left panel) in the absence or presence of *Eco*SSB. After transcription, the mixtures were split further into 3 samples: a control sample with no additions, a sample to which RNase H was added to specifically degrade RNA in RNA: DNA hybrids, and a third sample to which Nuclease S1 was added to degrade single-stranded nucleic acids. In the absence of *Eco*SSB, both the DNA template and the RNA product are in RNA: DNA hybrids, since the RNA product is RNase H sensitive while the DNA-containing bands show altered mobility after RNase H treatment. In the presence of *Eco*SSB, a significant portion of the RNA product is RNase H resistant and therefore free, although an RNase sensitive band is present that corresponds to an intermediate RNA: DNA: SSB complex. Under these conditions, the DNA is in an SSB: DNA complex. These results indicate that *Eco*SSB stimulates transcription through template recycling.

To define regions of *Eco*SSB essential for vRNAP transcription activation on single-stranded templates, the inventors have tested the effect of human mitochondrial SSB (HmtSSB), which shows extensive sequence and structural homology to *Eco*SSB. The N-terminus of *Eco*SSB contains DNA binding and tetramerization determinants while the C-terminus is involved in interaction with other replication proteins. Hmt SSB has no effect on vRNAP transcription although it does not melt the promoter hairpin. Interestingly, preliminary results using mutant *Eco*SSBs and *Eco*SSB-Hmt SSB chimeras suggest that the C-terminal region of *Eco*SSB is essential for vRNAP transcriptional activation.

**Example 4**

**Characterization of mini-vRNAP transcription properties**

The initiation properties of the full length RNA polymerase and mini-vRNAP were compared at similar molar concentrations (FIG. 9A) using the catalytic autolabeling assay and two reaction conditions: 1- using a template containing +1C, the benzaldehyde derivative of GTP

and $\alpha^{32}$P-ATP, or 2- a template containing +1T, the benzaldehyde derivative of ATP and $\alpha^{32}$P-GTP. Comparison of the results in FIGS. 9B and 9C demonstrates that mini-vRNAP exhibits initiation properties similar to full-length vRNAP. In addition, both enzymes discriminate against dATP incorporation to the same extent. Mini-vRNAP does not synthesize abortive products when the first four nucleotides of the transcript are comprised of 50% or more G or C nucleotides.

The elongation and termination properties of both enzymes are compared in FIG. 10. Similar run-off and terminated transcripts are synthesized. Moreover, *Eco*SSB activates transcription by both enzymes to the same levels. This result indicates that, if there are any sites of specific contact between vRNAP and *Eco*SSB, they reside in the mini-vRNAP domain.

The sequence of the terminator signals for vRNAP present in the N4 genome include SEQ ID NOS: 21-26. The signals of SEQ ID NO:21 and 22 have been tested *in vitro* on single-stranded templates.

The rate of mini-vRNAP transcription has been compared to the rate of T7 RNA polymerase under the same conditions using the same DNA template. The template used was linearized pET11 containing the original T7 promoter and the N4 vRNAP P2 promoter that was introduced through cloning. The DNA template was denatured before performing transcription using N4 mini-vRNAP. The concentrations of T7 RNAP (Promega, Cat.#P2075) and mini-vRNAP were compared using SDS-PAGE. Transcription reactions contained 50 nM of polymerase, 100 nM of DNA template, 5X transcription buffer provided with the T7 RNAP, and 1 mM of each ATP, GTP and CTP and 0.1 mM of [$^{32}$P]- UTP (1 Ci/mmol). Each reaction mixture was split in two, and *E. coli* SSB was added to one half. The mixtures were incubated at 37°C and aliquots were taken at different time points. Transcription products were electrophoresed on a 6% sequencing gel and the amount of radioactively-labeled RNA was quantitated by phosphoimaging. The results showed that: (a) transcription of T7 RNAP was not affected by the presence of *E. coli* SSB and (b) N4 mini-vRNAP synthesized 1.5 to 5 fold more RNA in the presence of *Eco*SSB than T7 RNAP at different time points of incubation.

The optimal temperature for mini-vRNAP transcription is 37°C. It exhibits 70% activity at 30°C, 65% at 45°C, and only 20% at 50°C.

The average error frequency was estimated by determining the misincorporation frequency of each of four [$^{32}$P]-α NTPs into RNA products using template ssDNAs missing the corresponding template nucleotide in the transcribed region. The following values were obtained: 1/5x$10^4$ for misincorporation of G and U using "no C" (SEQ ID NO:10) and "no A" (SEQ ID NO:11) ssDNA templates, respectively; 1/4x$10^4$ for misincorporation of C using the "no G" (SEQ ID NO:12) template, and 1/2x$10^4$ for misincorporation of A using the "no T" (SEQ ID NO:13) template. For comparison, the average error frequency for T7 RNAP is 1/2x$10^4$ (Huang, *et al.*, 2000). Using the method for detection of mispair formation described by Huang, *et al.* (2000), no misincorporation by mini-vRNAP was detected.

The ability of mini-vRNAP to incorporate derivatized nucleotides was measured. Transcription by mini-vRNAP in the presence of 0.1-1 mM Digoxigenin-11-UTP (cat# 1209256, Roche), Biotin-16-UTP (cat# 1388908, Roche) or underivatized UTP, yielded comparable amounts of product RNA using "control" ssDNA (SEQ ID NO:9) as a transcription template. The product RNAs synthesized in the presence of derivatized UTP have higher molecular mass than those synthesized in the presence of underivatized UTP, and the difference corresponds to the mass difference of the UTPs used. Several derivatives (*i.e.* 2'Fluoro-ribonucleoside triphosphates, dideoxynucleoside triphosphates) are being tested. The fluorescent analog Fluorescein-12-UTP (Roche catalog #1427857) has been tested using a template which encodes a 51 nucleotide transcript containing a run of 4 Us, and a nucleotide mix containing ATP, CTP, GTP and Fluorescein-12-UTP only. Transcription was only 3% of that achieved with UTP, biotin-6-UTP or digoxigenin-11-UTP under the same reaction conditions. However, incorporation of the fluorescent analog at higher yields is expected to occur in the presence of underivatized UTP or on templates with other sequence compositions.

**Example 5**

**Sequence determinants of mini-vRNAP promoter binding**

The three N4 early promoters present in the N4 genome contain a pair of Cs separated by 4 nucleotides from the base of the 5 bp promoter stem. In the preferred promoter P2, these 4 bases are As and the Cs are followed by a T. Preferably, mini-vRNAP uses a 17 nucleotide promoter sequence located immediately upstream of the transcription initiation site. Promoters for N4 vRNA polymerase are described by Haynes *et al*, (1985) and Dai *et al.*, (1998), herein

incorporated by reference. vRNAP-promoter recognition and activity require specific sequences and a hairpin structure on the template strand. The vRNAP promoters of SEQ ID NOS: 27-29 assume a hairpin structure comprised of a 5-7 bp stem (the inverted repeats are underlined in Table 6) and 3 b purine-containing loop (shown in bold in Table 6). The -11 position corresponds to the center of the loop; +1 indicates the transcription start site.

TABLE 6

Promoter Sequences

| | | |
|---|---|---|
| **P1** | **-11** **+1**<br>3'-CAACG**AAG**CGTTGAATACCT-5' | SEQ ID NO:27 |
| **P2** | **-11** **+1**<br>3'-TTCTTCG**AGG**CGAAGAAAACCT-5' | SEQ ID NO:28 |
| **P3** | **-11** **+1**<br>3'-CGACG**AGG**CGTCGAAAACCA-5' | SEQ ID NO:29 |

Other possible vRNAP promoters of the current invention include a set of any inverted repeats forming a hairpin with a 2-7 bp long stem and 3-5 b loop having purines in the central and/ or next to the central position of the loop.

To study the sequence determinants of promoter binding, 20 base-long promoter oligonucleotides, containing the wild type vRNAP promoter P2 sequence and substituted at every position with a single 5-Iodo-dU, were used. Whenever substitutions were made in the stem, the corresponding pairing base was changed to A. These oligonucleotides were $^{32}P$ end-labeled and used to determine the enzyme's affinity for promoter DNAs by a filter binding assay and the ability to crosslink to mini-vRNAP upon UV irradiation at 320nm. A 20-base oligonucleotide with wild type promoter P2 sequence binds with a 1 nM Kd. Most oligonucleotides showed close to wild type affinity except for the oligonucleotides substituted at positions –11 (at the center of the loop) and –8, indicating that these positions are essential for promoter recognition (FIG. 11). Surprisingly, UV crosslinking was most effective at position –11, in spite of the low binding affinity, indicating a specific contact at this position to mini-vRNAP. Crosslinking was also observed to positions +1, +2 and +3, indicating non-specific contacts with this region of the template, since 5-Iodo-dU substituted oligonucleotides at these positions showed wild type binding affinity.

The effect of changes in the stem length of the hairpin on the ability of mini-vRNAP to bind P2 promoter DNA was analyzed. As shown above, wild type promoter P2 with a 5 bp stem has a Kd of 1nM (FIG. 12, top). The stem was shortened by removal of 3' bases as shown in FIG. 12 (left). The stem can be shortened by two base pairs without change in the binding affinity. If two or one loop-closing base pairs remain, the binding affinity of templates is still substantial (2-10 nM). This result, although surprising, is not unexpected since it has been shown that the oligonucleotide 3'd(CGAGGCG)5' forms an unusually stable minihairpin (Yoshizawa, *et al.*, 1997). No binding is observed if one more nucleotide is removed and the loop cannot form. These results indicate that formation of a loop is essential for vRNAP-promoter recognition.

The effect of lengthening the stem by addition of 3' bases is shown in FIG. 12 (right). The stem can be lengthened by two base pairs without change in the binding affinity. On the other hand, base pairing at -2 reduces binding affinity by two orders of magnitude, with a further one order of magnitude reduction caused by base pairing at -1 and +1. These results indicate that single-strandedness of the template at positions -2, -1 and +1 is required for efficient template binding.

All three N4 early promoters present in the N4 genome contain a pair of Cs separated by 4 nucleotides from the base of the 5 bp promoter stem. In promoter P2, these 4 bases are As and the Cs are followed by a T. To identify the determinants of the site of transcription initiation, a series of templates were constructed with a single C placed at different distances from position -11 of the hairpin by addition or deletion of the tract of As present at promoter P2 (FIG. 13). The affinity of mini-vRNAP for these promoters was measured by filter binding and transcription initiation was measured by catalytic autolabeling of mini-vRNAP. All templates showed similar binding affinities. However, only the template with a C positioned 12 bases downstream from the center of the hairpin was able to support transcription initiation. This result indicates that mini-vRNAP utilizes this position as the transcription start site (+1).

## Example 6

### Identification of sequence motifs essential for mini-vRNAP activity

As shown in FIG. 2A, vRNAP contains the sequence $Rx_3Kx_{6-7}YG$, designated Motif B in the Pol I and Pol α DNA polymerases and the T7-like RNA polymerases. To determine the relevance of this motif to vRNAP activity, two mutants K670A and Y678F (SEQ ID NO:8) (position numbers in mini-vRNAP) were constructed by site-specific mutagenesis of mini-vRNAP. These two positions were chosen because, in T7-like RNA polymerases, the lysine is involved in nucleotide binding and the tyrosine in discrimination against deoxynucleoside triphosphates (Maksimova, *et al.*, 1991; Bonner, *et al.*, 1992; Osumi-Davis, *et al.*, 1992). The His-tagged Y678F mini-vRNAP gene (SEQ ID NO:7) differs from that of the mini-vRNAP domain sequence (SEQ ID NO:3) at two positions: nucleotide 2033 (A) was changed to a T, and nucleotide 2034 (T) was changed to a C.

These RNA polymerase mutants were cloned under pBAD control, purified and tested for their ability to bind to wild type promoters. Both mutant polymerases bound to promoter DNA with wild type affinities and crosslinked to 5-Iodo-dU substituted P2 DNA templates at positions −11 and +3 with wild type affinities (FIG. 14), indicating that these mutations do not affect promoter binding.

The mutant enzymes were tested for their ability to support run-off transcription. The wild type enzyme and Y678F enzyme (SEQ ID NO:8) displayed similar activities at both template excess and template-limiting conditions, while the K670A enzyme exhibited decreased activity under both conditions (FIG. 15). Under limiting template conditions, all three enzymes were activated by Eco SSB (right panel). However, the Y678F enzyme showed reduced discrimination between ribo- and deoxyribonucleoside triphosphates.

The initiation properties of the three enzymes were compared using catalytic autolabeling (FIG. 16). The K670A enzyme displays significantly reduced activity with the GTP derivative. The Y678F enzyme, in contrast to wild type polymerase, incorporates dATP as efficiently as rATP in a single round of phosphodiester bond formation.

Therefore, the behavior of the K670A and Y678F mutant enzymes indicates that Motif B is involved in catalysis, with the lysine probably required for NTP binding and the tyrosine

responsible for dNTP discrimination. These results suggest that, despite its lack of extensive sequence similarity, vRNAP is a Class II T7-like RNA polymerase. Results of recent experiments revealed the location of the two carboxylates (aspartates) involved in catalysis.

### Example 7

### Development of an *in vivo* system using mini-vRNAP and N4 vRNAP promoters for *in vivo* expression of RNAs and proteins

Plasmid templates were constructed with a reporter gene (α-peptide of ß-galactosidase) cloned under the control of vRNAP promoter P2 present in either of two orientations (FIG. 17B). The reporter construct was generated by cloning a cassette into plasmid pACYC177, which was obtained from New England Biolabs. The cassette contains an approximately 30 bp long fragment originating from pT7Ac (purchased from United States Biochemical), a N4 promoter, and sequence encoding the alpha fragment of lacZ (lacZ'). The N4 promoter and lacZ' were generated by oligonucleotide annealing and PCR™ amplification, respectively. This cassette replaces the pACY177 sequence located between the cleavage sites for restriction enzymes ApaLI and BamHI. These reporter plasmids and recombinant full-length or mini-vRNAP expressing plasmids were introduced into *E. coli* DH5α (ΔM15), a strain that encodes the ß-galactosidase ω-peptide. Expression of the reporter gene (α–peptide) in this strain results in the synthesis of active ß-galactosidase and consequent production of blue colonies on X-gal plates. Transcription of α-peptide by full-length and mini-vRNAP was assayed on inducing-Xgal media and shown in FIG. 17A. Induction of full-length polymerase results in small colonies with no ß-galactosidase activity. This is not surprising since full-length vRNAP is degraded in these cells (FIG. 17C). In contrast, induction of mini-vRNAP led to detectable levels of the protein (FIG. 17C) and to ß-galactosidase activity only from the plasmid containing promoter P2 in the proper orientation (FIG. 17A). These results indicate that this system will be suitable for *in vivo* expression of RNAs and proteins under mini-N4 vRNAP promoter control.

* * * * * * * * * * *

All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both

chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

## REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

EPA 320,308
EPA 329,822
GB 2202,328
PCT Application No. US87/00880
PCT Application No. US89/01025
U.S. Patent No. 6,218,145
U.S. Patent No. 4,682,195
U.S. Patent No. 4,683,195
U.S. Patent No. 4,683,202
U.S. Patent No. 4,684,611
U.S. Patent No. 4,797,368
U.S. Patent No. 4,800,159
U.S. Patent No. 4,883,750
U.S. Patent No. 4,946,773
U.S. Patent No. 4,952,500
U.S. Patent No. 5,082,592
U.S. Patent No. 5,139,941
U.S. Patent No. 5,279,721
U.S. Patent No. 5,302,523
U.S. Patent No. 5,322,783
U.S. Patent No. 5,354,855
U.S. Patent No. 5,384,253
U.S. Patent No. 5,464,765
U.S. Patent No. 5,538,877
U.S. Patent No. 5,538,880
U.S. Patent No. 5,550,318
U.S. Patent No. 5,563,055
U.S. Patent No. 5,580,859
U.S. Patent No. 5,589,466
U.S. Patent No. 5,591,616

U.S. Patent No. 5,610,042
U.S. Patent No. 5,656,610
U.S. Patent No. 5,670,488
U.S. Patent No. 5,672,344
U.S. Patent No. 5,693,489
U.S. Patent No. 5,702,932
U.S. Patent No. 5,736,524
U.S. Patent No. 5,780,448
U.S. Patent No. 5,789,215
U.S. Patent No. 5,869,320
U.S. Patent No. 5,945,100
U.S. Patent No. 5,981,274
U.S. Patent No. 5,994,136
U.S. Patent No. 5,994,624
U.S. Patent No. 5610287
U.S. Patent No. 6,013,516
U.S. Patent No. 6,046,173
WO 88/10315
WO 89/06700
WO 90/07641
WO 94/09699
WO 95/06128

Abravaya, K. and Rothman-Denes, L.B. (1990) N4 RNA polymerase II sites of transcription initiation. J. Mol. Biol. 211: 359-372.

Angel *et al.*, Cell, 49:729, 1987b.

Angel *et al.*, Mol. Cell. Biol., 7:2256, 1987a.

Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988

Archambault, J. and Friesen, J.D. (1993) Genetics of eukaryotic RNA polymerases I, II and III. Microbiol. Rev. 57: 703-724.

Atchison and Perry, Cell, 48:121, 1987.

Baichwal and Sugden, In: Gene Transfer, Kucherlapati R, ed., New York, Plenum Press, pp. 117-148, 1986.

Banerji *et al.*, Cell, 27:299, 1981.

Banerji *et al.*, Cell, 35:729, 1983.

Benvenisty and Neshif, Proc. Nat. Acad. Sci. USA, 83:9551-9555, 1986.

Berkhout *et al.*, Cell, 59:273, 1989.

Berzal-Herranz *et al., Genes and Devel.,* 6:129-134, 1992.

Blanar *et al.*, EMBO J., 8:1139, 1989.

Bodine and Ley, EMBO J., 6:2997, 1987.

Bonner, G., Patra, D., Lafer, E. M., and Sousa R.(1992) Mutations in T7 RNA polymerase that support the proposal for a common polymerase active site structure. EMBO J. 11: 3767-3775.

Boshart *et al.*, Cell, 41:521, 1985.

Bosze *et al.*, EMBO J., 5:1615, 1986.

Braddock *et al.*, Cell, 58:269, 1989.

Bulla and Siddiqui, J. Virol., 62:1437, 1986.

Butler and Chamberlin, *J. Biol. Chem., 257*: 5772-5778, 1982.

Campbell and Villarreal, Mol. Cell. Biol., 8:1993, 1988.

Campere and Tilghman, Genes and Dev., 3:537, 1989.

Campo *et al.*, Nature, 303:77, 1983.

Capaldi *et al., Biochem. Biophys. Res. Comm.*, 76:425, 1977.

Carter and Flotte, Ann. N.Y. Acad. Sci., 770:79-90, 1995.

Cech *et al.*, "*In vitro* splicing of the ribosomal RNA precursor of Tetrahymena: involvement of a guanosine nucleotide in the excision of the intervening sequence," *Cell*, 27:487-496, 1981.

Celander and Haseltine, J. Virology, 61:269, 1987.

Celander *et al.*, J. Virology, 62:1314, 1988.

Cermakian, N., Ikeda, T.M., Cedergren, R., and Gray, M.W. (1996) Sequences homologous to the yeast mitochondrial and bacteriophage T3 and T7 RNA polymerases are widespread throughout the eukaryotic lineage. Nuc. Acids Res. 24: 648-654.

Chamberlin and Ryan, In: The Enzymes. San Diego, CA, Academic Press, 15: 87-108, 1982

Chandler *et al.*, Cell, 33:489, 1983.

Chang *et al.*, "Foreign gene delivery and expression in hepatocytes using a hepatitis B virus vector," *Hepatology*, 14:134A, 1991.

Chang *et al.*, Mol. Cell. Biol., 9:2153, 1989.

Chase, J.W. and Williams, K.R. (1986) Single-stranded DNA binding proteins required for DNA replication. Ann. Rev. Biochem. 55: 130-136.

Chatterjee *et al.*, Proc. Natl. Acad. Sci. USA., 86:9114, 1989.

Cheetham, G. M. T. and Steitz, T. A. (2000) Insights into transcription: structure and function of single-subunit DNA-dependent RNA polymerases. Curr. Op. In Struc. Biol. 10: 117-123.

Cheetham, G. M., Jeruzalmi, D. and Steitz, T. A. 1999. Structural basis for initiation of transcription from an RNA polymerase-promoter complex. Nature 399: 80-83.

Chen and Okayama, Mol. Cell Biol., 7:2745-2752, 1987.

Choi *et al.*, Cell, 53:519, 1988.

Chowrira *et al.*, "*In vitro* and *in vivo* comparison of hammerhead, hairpin, and hepatitis delta virus self-processing ribozyme cassetyes," *J. Biol. Chem.*, 269:25856-25864, 1994.

Chowrira *et al.*, *Biochemistry*, 32:1088-1095, 1993.

Clark, Voulgaropoulou, Fraley, and Johnson, "Cell lines for the production of recombinant adeno-associated virus," *Human Gene Therapy*, 6:1329-1341, 1995.

Coetze *et al.*, 1994 Genes & Develop. 8, 1575.

Coffin, "Retroviridae and their replication," *In: Virology*, Fields *et al.* (eds.), New York: Raven Press, pp. 1437-1500, 1990.

Costa *et al.*, Mol. Cell. Biol., 8:81, 1988.

Couch *et al.*, "Immunization with types 4 and 7 adenovirus by selective infection of the intestinal tract," *Am. Rev. Resp. Dis.*, 88:394-403, 1963.

Cramer, P., Bushnell, D. A., and Kornberg, R. D. (2001) Structural basis of transcription: RNA polymerase II at 2.8 Å resolution Sciencexpress, www.sciencexpress.org. 19 April

Cramer, P., Bushnell, D. A., Fu, J., Gnatt, A. L., Maier-Davis, B., Thompson, N. E., Burgess, R. R., Edwards, A. M., David, P. R., and Kornberg, R. D. 2000. Science 288: 640-649.

Cripe *et al.*, EMBO J., 6:3745, 1987.

Culotta and Hamer, Mol. Cell. Biol., 9:1376, 1989.

Dai, X and Rothman-Denes, L. B. 1998. Sequence and DNA structural determinants of N4 virion RNA polymerases-promoter recognition. Genes Devepmnt. 12:2782-2790.

Dandolo *et al.*, J. Virology, 47:55, 1983.

De Villiers *et al.*, Nature, 312:242, 1984.

Delarue, M., Poch, O., Tordo, N., Moras, D., and Argos, P. (1990) An attempt to unify the structure of polymerases. Protein Engineering. 3: 461-467.

Deschamps *et al.*, Science, 230:1174, 1985.

Di Chiara *et al.*, *Trends Pharmacol. Sci.*, 13:185, 1992.

Dubensky *et al.*, Proc. Nat. Acad. Sci. USA, 81:7529-7533, 1984.

Dunn *et al.*, *Nature New Biology, 230*: 94-96, 1971

Edbrooke *et al.*, Mol. Cell. Biol., 9:1908, 1989.

Edlund *et al.*, Science, 230:912, 1985.

Elliott, Hynansky, Inturrisi, "Dextromethorphan attenuates and reverses analgesic tolerance to morphine," *Pain*, 59:361-368, 1994.

Falco, S. C. and Rothman-Denes, L. B. 1979. Bacteriophage N4-induced transcribing activities in *E. coli*: I. Detection and characterization in cell extracts. Virology 95: 454-465.

Falco, S. C., Vander Laan, K., and Rothman-Denes, L. B. 1977. Virion-associated RNA polymerase required for bacteriophage N4 development. Proc. Natl. Acad.Sci. (USA) 74: 520-523.

Falco, S. C., Zehring, W. A., and Rothman-Denes, L. B. 1980. DNA-dependent RNA polymerase from bacteriophage N4 virions: purification and characterization. J. Biol. Chem. 255: 4339-4347.

Falco, S. C., Zivin, R., and Rothman-Denes, L. B. 1978. Novel template requirements of N4 virion RNA polymerase. Proc. Natl. Acad. Sci. (USA) 75: 3220-3224.

Faraldo et al., J. Bact., 174: 7458-7462, 1992

Fechheimer *et al.*, Proc. Natl. Acad. Sci. USA, 84:8463-8467, 1987.

Feng and Holland, Nature, 334:6178, 1988.

Ferkol *et al.*, FASEB J., 7:1081-1091, 1993.

Firak and Subramanian, Mol. Cell. Biol., 6:3667, 1986.

Foecking MK, Hofstetter H. *Gene*. 45(1):101-105, 1986.

Foley, "Opioid analgesics in clinical pain management. In: *Handbook of Experimental Pharmacology*, Herz, (Ed.), Vol. Vol. 104/II: Opioids II., Springer-Verlag, Berlin, pp. 693-743, 1993.

Forster and Symons, "Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites," *Cell*, 49:211-220, 1987.

Fraley *et al.*, Proc. Natl. Acad. Sci. USA, 76:3348-3352, 1979.

Frohman, *In*: *PCR Protocols: A Guide To Methods And Applications*, Academic Press, N.Y., 1990.

Fujita *et al.*, Cell, 49:357, 1987.

Gerlach *et al.*, "Construction of a plant disease resistance gene from the satellite RNA of tobacco rinspot virus," *Nature (London)*, 328:802-805, 1987.

Ghosh and Bachhawat, In: Liver diseases, targeted diagnosis and therapy using specific receptors and ligands, (Wu G, Wu C ed.), New York: Marcel Dekker, pp. 87-104, 1991.

Gilles *et al.*, Cell, 33:717, 1983.

Gloss *et al.*, EMBO J., 6:3735, 1987.

Glucksmann, M. A., Markiewicz, P., Malone, C., and Rothman-Denes, L. B. 1992. Specific sequences and a hairpin structure in the template strand are required for N4 virion RNA polymerase-promoter recognition. Cell 70, 491-500.

Glucksmann-Kuis, A.M., Dai, X., Markiewicz, P. M. and Rothman-Denes, L. B.1996. *E. coli* SSB activation of N4 virion RNA polymerase: specific stabilization of an essential DNA hairpin required for promoter recognition. Cell 84, 147-154.

Godbout *et al.*, Mol. Cell. Biol., 8:1169, 1988.

Gomez-Flores and Weber, "Differential effects of buprenorphine and morphine on immune and neuroendocrine functions following acute administration in the rat mesencephalon periaqueductal gray," *Immunopharm,m, 48*:145-156, 2000.

Goodbourn and Maniatis, Proc. Natl. Acad. Sci. USA, 85:1447, 1988.

Goodbourn *et al.*, Cell, 45:601, 1986.

Gopal, Mol. Cell Biol., 5:1188-1190, 1985.

Graham and Prevec, "Adenovirus-based expression vectors and recombinant vaccines," *Biotechnology*, 20:363-390, 1992.

Graham and Prevec, "Manipulation of adenovirus vectors," In. Gene Transfer and Expression Protocols, Murray, E. J., ed., Humana, New Jersey, vol. 7, 109-128, 1991.

Graham and Van Der Eb, Virology, 52:456-467, 1973.

Greene *et al.*, Immunology Today, 10:272, 1989.

Gross, C.A., Chan, C., Dombroski, A., Gruber, T., Sharp, M., Tupy, J., and Young, B. (1998) The functional and regulatory roles of sigma factors in transcription. In: Mechanisms of Transcription. Cold Spring Harbor Symp. Quant. Biol. 63: 141-156.

Grosschedl and Baltimore, Cell, 41:885, 1985.

Guzman, L.M. *et al.* (1995) J. Bact. 177: 4121-4130.

Hartmann et al., Nucl. Acids Res., 19: 5957-5964, 1991

Hartmann et al., Biochem, 69: 1097-1104, 1987

Hartmann, G.R., Biebricker, C., Glaser, S. J., Grosse, F., Katzameyer, M., Lindner, A.J., Mosig, H., Nasheuer, H.P., Rothman-Denes, L.B., Schaffner, A.R., Schneider, G., Stetter, K-D., and Thomm, M . 1988. Initiation of transcription- a general tool for affinity labelling of RNA polymerases by autocatalysis. Biol. Chem. Hoppe-Seyler 369: 775-788.

Haseloff and Gerlach, "Simple RNA enzymes with new and highly specific endoribonuclease activities," *Nature*, 334:585-591, 1988.

Haslinger and Karin, Proc. Natl. Acad. Sci. USA., 82:8572, 1985.

Hauber and Cullen, J. Virology, 62:673, 1988.

Hausmann *Current Topics in Microbiology and Immunology, 75*: 77-109, 1976

Haynes, L. L. and Rothman-Denes, L. B. 1985. N4 virion RNA polymerase sites of transcription initiation. Cell 41: 597-605.

Hedtke, B., Borner, T., and Weihe, A. (1997) Mitochondrial and chloroplast phage-type RNA polymerases in Arabidopsis. Science. 277: 809-811.

Hen *et al.*, Nature, 321:249, 1986.

Hensel *et al.*, Lymphokine Res., 8:347, 1989.

Herr and Clarke, Cell, 45:461, 1986.

Higuchi, *et al., Biotechnology* 10:413-417 1992.

Hirochika *et al.*, J. Virol., 61:2599, 1987.

Hirsch *et al.*, Mol. Cell. Biol., 10:1959, 1990.

Hochschild, A. and Dove, S.L. (1998) Protein-protein contacts that activate and repress prokaryotic transcription. Cell 92:597-600.

Holbrook *et al.*, Virology, 157:211, 1987.

Horlick and Benfield, Mol. Cell. Biol., 9:2396, 1989.

Huang *et al.*, Cell, 27:245, 1981.

Huang, J., Brieba, L. G., and Sousa, R. (2000) Misincorporation by Wild-Type and Mutant T7 RNA Polymerases: Identification of Interactions That Reduce Misincorporation Rates by Stabilizing the Catalytically Incompetent Open Conformation Biochemistry, 38: 11571-11580.

Hughes, J.; Smith, T.W.; Kosterlitz, H.; Fothrgill, L.; Morgan, B. and Morris, H. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 1975, *258*: 577-579.

Hwang *et al.*, Mol. Cell. Biol., 10:585, 1990.

Imagawa *et al.*, Cell, 51:251, 1987.

Imbra and Karin, Nature, 323:555, 1986.

Imler *et al.*, Mol. Cell. Biol., 7:2558, 1987.

Imperiale and Nevins, Mol. Cell. Biol., 4:875, 1984.

Jakobovits *et al.*, Mol. Cell. Biol., 8:2555, 1988.

Jameel and Siddiqui, Mol. Cell. Biol., 6:710, 1986.

Jaynes *et al.*, Mol. Cell. Biol., 8:62, 1988.

Johnson *et al.*, Mol. Cell. Biol., 9:3393, 1989.

Jones and Shenk, "Isolation of deletion and substitution mutants of adenovirus type 5," *Cell*, 13:181-188, 1978.

Jorgensen et al., J. Biol. Chem., 266: 645-655, 1991.

Joyce, "RNA evolution and the origins of life," *Nature*, 338:217-244, 1989.

Kadesch and Berg, Mol. Cell. Biol., 6:2593, 1986.

Kaneda *et al.*, Science, 243:375-378, 1989.

Karin *et al.*, Mol. Cell. Biol., 7:606, 1987.

Kase *et al.*, A potent inhibitor ofprotein kinase C from microbial origin," *J. Antibiot.*, (8):1059-1065, 1986

Katinka *et al.*, Cell, 20:393, 1980.

Katinka *et al.*, Nature, 290:720, 1981.

Kato *et al*, J. Biol. Chem., 266:3361-3364, 1991.

Kawamoto *et al.*, Mol. Cell. Biol., 8:267, 1988.

Kazmierczak, K.M.2001. PhD thesis. The University of Chicago.

Kiledjian *et al.*, Mol. Cell. Biol., 8:145, 1988.

Kim and Cech, "Three dimensional model of the active site of the self-splicing rRNA precursor of Tetrahymena," *Proc. Natl. Acad. Sci. USA*, 84:8788-8792, 1987.

Klamut *et al.*, Mol. Cell. Biol., 10:193, 1990.

Klein *et al.*, Nature, 327:70-73, 1987.

Koch *et al.*, Mol. Cell. Biol., 9:303, 1989.

Korsten *et al.*, *J. Gen. Virol.*, *43*: 57-73, 1975

Kriegler and Botchan, In: Eukaryotic Viral Vectors, Y. Gluzman, ed., Cold Spring Harbor: Cold Spring Harbor Laboratory, NY, 1982.

Kriegler and Botchan, Mol. Cell. Biol., 3:325, 1983.

Kriegler *et al.*, Cell, 38:483, 1984a.

Kriegler *et al.*, Cell, 53:45, 1988.

Kriegler *et al.*, In: Cancer Cells 2/Oncogenes and Viral Genes, Van de Woude *et al.*, eds, Cold Spring Harbor, Cold Spring Harbor Laboratory, 1984b.

Kriegler *et al.*, In: Gene Expression, D. Hamer and M. Rosenberg, eds., New York: Alan R. Liss, 1983.

Kuhl *et al.*, Cell, 50:1057, 1987.

Kunz *et al.*, Nucl. Acids Res., 17:1121, 1989.

Larsen *et al.*, Proc. Natl. Acad. Sci. USA., 83:8283, 1986.

Laspia *et al.*, Cell, 59:283, 1989.

Latimer *et al.*, Mol. Cell. Biol., 10:760, 1990.

Le Gal La Salle *et al.*, "An adenovirus vector for gene transfer into neurons and glia in the brain," *Science*, 259:988-990, 1993.

Lee *et al.*, Nature, 294:228, 1981.

Lee, Tomasetto, Sager, Proc. Natl. Acad. Sci. USA, 88:2825, 1991.

Levinson *et al.*, Nature, 295:79, 1982.

Lieber and Strauss, "Selection of efficient cleavage sites in target RNAs by using a ribozyme expression library." *Mol. Cell. Biol.*, 15: 540-551, 1995.

Lin *et al.*, Mol. Cell. Biol., 10:850, 1990.

Luria *et al.*, EMBO J., 6:3307, 1987.

Lusky and Botchan, Proc. Natl. Acad. Sci. USA., 83:3609, 1986.

Lusky *et al.*, Mol. Cell. Biol., 3:1108, 1983.

Lutz *et al.*, *J. Receptor Res.*, 12:267, 1992.

Majors and Varmus, Proc. Natl. Acad. Sci. USA., 80:5866, 1983.

Maksimova, T. G., Mustayev, A. A., Zaychikov, E. F., Lyakhov, D. L., Tunitskaya, V. L., Akbarov, A. K., Luchin, S.V., Rechinsky, V. O., Chernov, B. K., and Kochetkov, S. N. (1991) Lys631 residue in the active site of the bacteriophage T7 RNA polymerase. Affinity labeling and site-directed mutagenesis. Eur J Biochem. 195: 841-847.

Malone, C., Spellman, S., Hyman, D., and Rothman-Denes, L.B. 1988. Cloning and generation of a genetic map of bacteriophage N4 . Virology 162: 328-336.

Mann *et al.*, "Mammalian protein serine/threonine phosphatase 2C: cDNA cloning and comparative analysis of amino acid sequences," *Biochim. Biophys. Acta*, 1130:100-104, 1992.

Markiewicz, P., Malone, C., Chase, J. W. and Rothman-Denes, L. B. 1992, *E. coli* single-stranded DNA binding (SSB) protein is a supercoiled-template dependent transcriptional activator of N4 virion RNA polymerase. Genes and Dev., 6: 2010-2019.

Martin and Coleman, Biochemistry, 26: 2690-2696, 1987

McGraw et al., Nucl. Acid. Res., 13: 6753-6766, 1985

McLaughlin, Collis, Hermonat, and Muzyczka, "Adeno-Associated Virus General Transduction Vectors: Analysis of Proviral Structures," *J. Virol.*, 62:1963-1973, 1988.

McNeall *et al.*, Gene, 76:81, 1989.

Michel and Westhof, "Modeling of the three-dimensional architecture of group I catalytic introns based on comparative sequence analysis," *J. Mol. Biol.*, 216:585-610, 1990.

Miksicek *et al.*, Cell, 46:203, 1986.

Miller, A.A., Wood, D., Ebright, R.E., and Rothman-Denes, L.B. (1997) RNA polymerase ß' subunit: a target of DNA binding-independent activation. Science. 275: 1655-1657.

Miller, *Curr. Top. Microbiol. Immunol.*, 158:1, 1992.

Miyomoto and Takemore, "Inhibition of naloxone-precipitated withdrawal jumping by i.c.v. and i.t. administration of saline in morphine-dependent mice," *Life Sci.*, 52(13):1129-1134, 1993b.

Miyomoto and Takemore, "Relative involvement of supraspinal and spinal mu opioid receptors in morphine dependence in mice," *Life Sci.*, 52(12):1039-1044, 1993a.

Mohr *et al.*, 1994 Nature 370, 147.

Mordacq and Linzer, Genes and Dev., 3:760, 1989.

Moreau *et al.*, Nucl. Acids Res., 9:6047, 1981.

Muesing *et al.*, Cell, 48:691, 1987.

Narita, Nartia, Mizoguchi, Tseng, "Inhibition of Protein Kinase C, but not of Protein Kinase A, blocks the development of acute antinociceptive tolerance to an intrathecally administered μ-opioid receptor agonist in the mouse," *European Pharmacology*, 280:R1-R3, 1995.

Ng *et al.*, Nuc. Acids Res., 17:601, 1989.

Nicolas and Rubinstein, "Retroviral vectors," *In: Vectors: A survey of molecular cloning vectors and their uses,* Rodriguez and Denhardt (eds.), Stoneham: Butterworth, 494-513, 1988.

Nicolau and Sene, Biochim. Biophys. Acta, 721:185-190, 1982.

Nicolau *et al.*, Methods Enzymol., 149:157-176, 1987.

Olson *et al.*, *Peptides,* 10:1253, 1988.

Ondek *et al.*, EMBO J., 6:1017, 1987.

Ornitz *et al.*, Mol. Cell. Biol., 7:3466, 1987.

Osumi-Davis, P. A., de Aguilera, M. C., Woody, R. W., and Woody, A. Y. (1992) Asp537, Asp812 are essential and Lys631, His811 are catalytically significant in bacteriophage T7 RNA polymerase activity. J Mol Biol.226:37-45.

Palmiter *et al.*, Nature, 300:611, 1982.

Palukaitis *et al.*, "Characterization of a viroid associated with avacado sunblotch disease," *Virology*, 99:145-151, 1979.

Paule, M. and White, R. J. (2000) Transcription by RNA polymerases I and III. Nuc. Acids Res. 28:1283-1298.

Pech *et al.*, Mol. Cell. Biol., 9:396, 1989.

Perales *et al.*, Proc. Natl. Acad. Sci. 91:4086-4090, 1994.

Perez-Stable and Constantini, Mol. Cell. Biol., 10:1116, 1990.

Perriman *et al.*, "Extended target-site specificity for a hammerhead ribozyme," *Gene,* 113:157-163, 1992.

Pert and Snyder, "Opiate receptor; demonstration in nervous tissue," *Science,* 179:1011-1014, 1973.

Picard and Schaffner, Nature, 307:83, 1984.

Pick, Roques, Gacel, Pasternak, "Supraspinal $mu_2$ receptors mediate spinal/supraspinal morphine synergy," *Eur. J. Pharmacol.*, 220:275-277, 1992a.

Pinkert *et al.*, Genes and Dev., 1:268, 1987.

Ponta *et al.*, Proc. Natl. Acad. Sci. USA., 82:1020, 1985.

Porton *et al.*, Mol. Cell. Biol., 10:1076, 1990.

Potter *et al.*, Proc. Nat. Acad. Sci. USA, 81:7161-7165, 1984.

Prody *et al.*, "Autolytic processing of dimeric plant virus satellite RNA." *Science,* 231:1577-1580, 1986.

Puhler, G., Leffers, H., Gropp, F., Palm, P., Klenk, H.-P., Lottspeich, F., Garrett, R.A., and Zillig, W. (1989) Archaebacterial DNA-dependent RNA polymerases testify to the evolution of the eukaryotic nuclear genome. Proc. Natl. Acad. Sci. USA. 86: 4569-4573

Queen and Baltimore, Cell, 35:741, 1983.

Quinn *et al.*, Mol. Cell. Biol., 9:4713, 1989.

Radler *et al.*, Science, 275:810-814, 1997.

Rashtchian and Mackey, "Labeling and Detection of Nucleic Acids," in "Nonradioactive Labeling and Detection of Biomolecules," C. Kessler, Ed., Springer-Verlag, New York, 1992, pp. 70-84

Record, M.T., Reznikoff, W.S., and Schlax, P.J. (1995) *E. coli* RNA polymerase (E-sigma70), promoters and the kinetics of the steps of transcription initiation, In *E. coli* and Salmonella typhimurium: Cell and Molecular Biology 1:792-821. F. Neidhardt ed. ASM

Redondo *et al.*, Science, 247:1225, 1990.

Reinhold-Hurek and Shub, "Self-splicing introns in tRNA genes of widely divergent bacteria," *Nature,* 357:173-176, 1992.

Reisman and Rotter, Mol. Cell. Biol., 9:3571, 1989.

Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990.

Resendez Jr. *et al.*, Mol. Cell. Biol., 8:4579, 1988.

Ripe *et al.*, Mol. Cell. Biol., 9:2224, 1989.

Rippe *et al.*, Mol. Cell Biol., 10:689-695, 1990.

Rittling *et al.*, Nucl. Acids Res., 17:1619, 1989.

Roeder, R. (1996) The role of general transcription factors in transcription by RNA polymerase II. Trends Biochem. Sci. 21: 327-335.

Rong, M., He, B., McAllister, W. T. and Durbin, R. K. (1998) Promoter specificity determinants of T7 RNA polymerase Proc. Natl. Acad. Sci. USA 95: 515-519.

Rosen *et al.*, Cell, 41:813, 1988.

Rossi, Pasternak, Bodnar, "Synergistic brainstem interactons for morphine analgesia," *Brain Res.*, 624: 171-180, 1993.

Rothman-Denes, L. B., Dai, X., Davydova, E., Carter, R., and Kazmierczak, K. 1999. Transcriptional Regulation by DNA Structural Transitions and Single-Stranded DNA Binding Proteins. 63rd Cold Spring Harbor Symp. Quant. Biol. 63: 63-73.

Roux *et al.*, "A versatile and potentially general approach to the targeting of specific cell types by retroviruses: Application to the infection of human cells by means of major histocompatibility complex class I and class II antigens by mouse ecotropic murine leukemia virus-derived viruses," *Proc. Natl. Acad. Sci. USA*, 86:9079-9083, 1989.

Running. J. A. *et al.*, BioTechniques 8:276-277, 1990.

Sakai *et al.*, Genes and Dev., 2:1144, 1988.

Saldanha *et al.*, 1993 FASEB. J. 7, 15.

Sambrook, Fritsch, Maniatis, In: Molecular Cloning: A Laboratory Manual 2 rev.ed., Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.

Sanders, G.M., Kassavetis, G.A., and Geiduschek, E.P. (1997) Dual targets of a transcriptional activator that tracks on DNA. EMBO Journal. 16: 3124-3132.

Sarver *et al.*, "Ribozymes as potential anti-HIV-1 therapeutic agents," *Science*, 247:1222-1225, 1990.

Satake *et al.*, J. Virology, 62:970, 1988.

Scanlon *et al.*, "Ribozyme-mediated cleavages of c-fos mRNA reduce gene expression of DNA synthesis enzymes and metallothionein," *Proc Natl Acad Sci USA*, 88:10591-10595, 1991.

Schaffner *et al.*, J. Mol. Biol., 201:81, 1988.

Searle *et al.*, Mol. Cell. Biol., 5:1480, 1985.

Shadel, G. S. and Clayton, D. A. (1993) Mitochondrial transcription initiation, variation and conservation. J. Biol. Chem. 268: 16083-16086.

Sharma, Klee, Nirenberg, "Opiate-dependent modution of adenylate cyclase,". *Proc. Natl. Acad. Sci. U.S.A.*, 74:3365-3369, 1977.

Sharp and Marciniak, Cell, 59:229, 1989.

Sharp and Yaksh, "Pain killers of the immune system," *Nature Medicine,* 3:831-832, 1997.

Shaul and Ben-Levy, EMBO J., 6:1913, 1987.

Shelling and Smith, "Targeted integration of transfected and infected adeno-associated virus vectors containing the neomycin resistance gene," *Gene Therapy,* 1:165-169, 1994.

Sherman *et al.*, Mol. Cell. Biol., 9:50, 1989.

Simon, *Medicinal Res. Rev.*, 11:357, 1991.

Sioud *et al.*, "Preformed ribozyme destroys tumour necrosis factor mRNA in human cells," *J Mol. Biol.*, 223:831-835, 1992.

Sleigh and Lockett, J. EMBO, 4:3831, 1985.

Sousa, R. (1996) Structural and mechanistic relationships between nucleic acid polymerases. Trends in Biochem. Sci. 21: 186-190.

Sousa, R., Chung, Y., Rose, J.P., and Wang, B.-C. (1993) Crystal strcuture of bacteriophage T7 RNA polymerase. Nature. 364: 593-599.

Spalholz *et al.*, Cell, 42:183, 1985.

Spandau and Lee, J. Virology, 62:427, 1988.

Spandidos and Wilkie, EMBO J., 2:1193, 1983.

Stephens and Hentschel, Biochem. J., 248:1, 1987.

Stuart *et al.*, Nature, 317:828, 1985.

Sullivan and Peterlin, Mol. Cell. Biol., 7:3315, 1987.

Swartzendruber and Lehman, J. Cell. Physiology, 85:179, 1975.

Sweetser, D., Nonet, M., and Young, R.A. (1987) Prokaryotic and eukaryotic RNA polymerases have homologous core subunits. Proc. Natl. Acad. Sci. USA. 84: 1192-1196.

Symons, "Avacado sunblotch viroid: primary sequence and proposed secondary structure." *Nucl. Acids Res.*, 9:6527-6537, 1981.

Symons, "Small catalytic RNAs." *Annu. Rev. Biochem.*, 61:641-671, 1992.

Takebe *et al.*, Mol. Cell. Biol., 8:466, 1988.

Tavernier *et al.*, Nature, 301:634, 1983.

Taylor and Kingston, Mol. Cell. Biol., 10:165, 1990a.

Taylor and Kingston, Mol. Cell. Biol., 10:176, 1990b.

Taylor *et al.*, J. Biol. Chem., 264:15160, 1989.

Thiesen *et al.*, J. Virology, 62:614, 1988.

Thompson *et al.*, "Ribozymes in gene therapy." *Nature Medicine*, 1:277-278, 1995.

Top *et al.*, "Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to adenovirus type 7," *J. Infect. Dis.*, 124:155-160, 1971.

Towle *et al.*, *J. Biol. Chem.*, *250*:1723-1733, 1975

Tronche *et al.*, Mol. Biol. Med., 7:173, 1990.

Tronche *et al.*, Mol. Cell. Biol., 9:4759, 1989.

Trudel and Constantini, Genes and Dev., 6:954, 1987.

Tur-Kaspa *et al.*, Mol. Cell Biol., 6:716-718, 1986.

Tyndall *et al.*, Nuc. Acids. Res., 9:6231, 1981.

Ueda, Miyamae, Hayashi, Watanabe, Fukushima, Sasaki, Iwamura, Misu, "Protein kinase C involvement in homologous desensitization of α-opioid receptor coupled to Gi1-phosoholipase C activation in xenopus oocytes," *Journal of Neuroscience*, 15:7485-7499, 1995.

Vannice and Levinson, J. Virology, 62:1305, 1988.

Vasseur *et al.*, Proc. Natl. Acad. Sci. USA., 77:1068, 1980.

Wagner *et al.*, Proc. Natl. Acad. Sci. 87(9):3410-3414, 1990.

Wagner *et al.*, Science, 260:1510-1513, 1993.

Walsh, Nienhuis, Samulski, Brown, Miller, Young, and Liu, "Phenotypic correction of Fanconi anemia in human hematopoietic cells with a recombinant adeno-associated virus vector," *J. Clin. Invest*, 94:1440-1448, 1994.

Wang and Calame, Cell, 47:241, 1986.

Wang, Johnson, Persico, Hawkins, Griffin, Uhl, "Human α opiate receptor: cDNA and genomic clones, pharmacologic characterization and chromosomal assignment," *FEBS Letters*, 338: 217-222, 1994.

Watt *et al.*, Proc. Natl Acad. Sci., 83(2): 3166-3170, 1986.

Weber *et al.*, Cell, 36:983, 1984.

Weinberger *et al.*, Mol. Cell. Biol., 8:988, 1984.

Wendt et al., Eur. J. Biochem., 191: 467-472, 1990

Whistler, J. and vonZastrow, M. (1998) Proc. Natl. Acad. Sci. 95, 9914-9.

Winoto and Baltimore, Cell, 59:649, 1989.

Wong *et al.*, Gene, 10:87-94, 1980.

Wu and Wu, Adv. Drug Delivery Rev., 12:159-167, 1993.

Wu and Wu, Biochem., 27:887-892, 1988.

Wu and Wu, J. Biol. Chem., 262:4429-4432, 1987.

Yang *et al.*, Proc. Natl. Acad. Sci USA, 87:9568-9572, 1990.

Yang, Chen, Trempe, "Characterization of cell lines that inducibly express the adeno-associated virus Rep proteins," *J. Virol*, 68:4847-4856, 1994.

Yoshizawa, S., Kawai, G., Watanabe, K., Miura, K., and Hirao, I. (1997) GNA trinucleotide loop sequences producing extraordinarily stable DNA mini hairpins. Biochemistry 36, 4761-4767.

Yuan and Altman, "Selection of guide sequences that direct efficient cleavage of mRNA by human ribonuclease P," *Science*, 263:1269-1273, 1994.

Yuan *et al.*, "Targeted cleavage of mRNA by human RNase P," *Proc. Natl. Acad. Sci. USA*, 89:8006-8010, 1992.

Yutzey *et al.*, Mol. Cell. Biol., 9:1397, 1989.

Zehring, W.A. and Rothman-Denes, L.B. (1983) Purification and characterization of coliphage N4 RNA polymerase II activity from infected cell extracts. J. Biol. Chem. 258: 8074-8080.

Zhang, G., Campbell, E. A., Minakhin, L., Richter, C., Severinov, K. and Darst, S. A. (1999) Crystal structure of Thermus aquaticus core RNA polymerase at 3.3.Å resolution. Cell 98: 811-824.

Zhou and Doetsch, Proc. Nat. Acad. Sci. USA, 90: 6601-6605, 1993

Zivin, R., Zehring, W. A., and Rothman-Denes, L. B. 1981. Transcriptional map of bacteriophage N4: location and polarity of N4 RNAs . J. Mol. Biol. 152: 335-356.

# CLAIMS

1. An isolated nucleic acid comprising a region encoding a polypeptide having an amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:15.

2. The nucleic acid of claim 1, wherein said nucleic acid comprises the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:14.

3. The nucleic acid of claim 1, wherein said nucleic acid is operatively linked to a promoter.

4. The nucleic acid of claim 3, wherein said promoter is an N4 vRNAP promoter set forth in SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:27, SEQ ID NO:28 or SEQ ID NO:29.

5. The nucleic acid of claim 3, wherein said promoter is a P2 sequence set forth in SEQ ID NO:16 or SEQ ID NO:28.

6. A recombinant host cell comprising a DNA segment encoding a N4 virion RNA polymerase.

7. The recombinant host cell of claim 6, wherein said DNA segment is a single-stranded DNA segment.

8. The recombinant host cell of claim 6, wherein said DNA segment is a double-stranded DNA segment.

9. The recombinant host cell of claim 6, wherein said DNA segment encodes a polypeptide having an amino acid sequence set forth in SEQ ID NO:4.

10. The recombinant host cell of claim 6, wherein said DNA segment encodes a polypeptide having an amino acid sequence set forth in SEQ ID NO:6.

11. The recombinant host cell of claim 6, wherein said cell is an *E. coli* cell.

12. A recombinant vector comprising a DNA segment encoding a N4 virion RNA polymerase polypeptide under the control of a promoter.

13. An isolated polynucleotide comprising a sequence identical or complementary to SEQ ID NO:1.

14. An isolated polynucleotide comprising a sequence identical or complementary to SEQ ID NO:3.

15. A purified N4 virion RNA polymerase comprising the polypeptide sequence of SEQ ID NO:2.

16. An isolated nucleic acid comprising a region encoding a polypeptide comprising at least 6 contiguous amino acids of the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8, wherein said polypeptide has RNA polymerase activity under appropriate reaction conditions.

17. The nucleic acid of claim 16, wherein said polypeptide comprises at least 20 contiguous amino acids of said amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8.

18. The nucleic acid of claim 17, wherein said polypeptide comprises at least 40 contiguous amino acids of the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8.

19. The nucleic acid of claim 18, wherein said polypeptide comprises at least 100 contiguous amino acids of the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8.

20. The nucleic acid of claim 16, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8.

21. The nucleic acid of claim 16, wherein said polypeptide has at least one histidine tag.

22. The nucleic acid of claim 16, wherein said polypeptide has a mutation at position Y678.

23. A method of making RNA comprising:

(a) obtaining a N4 virion RNA polymerase;

(b) obtaining DNA;

(c) admixing said RNA polymerase and said DNA; and

(d) culturing said RNA polymerase and said DNA under conditions effective to allow RNA synthesis.

24. The method of claim 23, further comprising synthesizing polynucleotides containing modified ribonucleotides or deoxyribonucleotides.

25. The method of claim 23, wherein said DNA is single-stranded DNA.

26. The method of claim 23, wherein said DNA is double-stranded DNA.

27. The method of claim 23, wherein said admixing occurs in a host cell.

28. The method of claim 27, wherein said host cell is an *E. coli* host cell.

29. The method of claim 23, wherein said RNA polymerase has the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:15.

30. The method of claim 29, wherein said RNA polymerase has the amino acid sequence set forth in SEQ ID NO:4.

31. The method of claim 23, wherein said RNA polymerase is a mutant of an RNA polymerase having the amino acid sequence set forth in SEQ ID NO:4 or SEQ ID NO:6.

32. The method of claim 31, wherein said mutant has a mutation at position number Y678.

33. The method of claim 32, wherein said mutant is histidine tagged.

34. The method of claim 23, wherein said RNA contains derivatized nucleotides.

35. The method of claim 23, further comprising using a promoter.

36. The method of claim 35, wherein said promoter is an N4 vRNAP promoter set forth in SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:27, SEQ ID NO:28 or SEQ ID NO:29.

37. The method of claim 36, wherein said promoter is a P2 sequence set forth in SEQ ID NO:16 or SEQ ID NO:28.

38. The method of claim 35, wherein the promoter comprises a set of inverted repeats forming a hairpin with a 2-7 base pair long stem and 3-5 base loop having purines in the central and/ or next to the central position of the loop.

39. The method of claim 35, wherein said promoter sequence is upstream of the transcription initiation site.

40. The method of claim 23, wherein step (c) is carried out at a pH of between 6 and 9.

41. The method of claim 40, wherein step (c) is carried out at a pH of between 7.5 and 8.5.

42. The method of claim 23, further comprising admixing $Mg^{+2}$ or $Mn^{+2}$.

43. The method of claim 42, comprising admixing $Mg^{+2}$.

44. The method of claim 23, further defined as carried out at a temperature of 25°C to 50°C.

45. The method of claim 44, further defined as carried out at a temperature of 30°C to 45°C.

46. The method of claim 45, further defined as carried out at a temperature of 32°C to 42°C.

47. The method of claim 23, further comprising the step of translation.

48. The method of claim 23, further comprising using a reporter gene.

49. The method of claim 48, wherein said reporter gene is an α-peptide of β-galactosidase.

50. The method of claim 23, wherein said admixing occurs *in vivo*.

51. The method of claim 23, wherein said admixing occurs *in vitro*.

52. The method of claim 23, further comprising admixing an *E. coli* single-stranded binding protein (*Eco*SSB), a SSB protein homologous to *Eco*SSB or another naturally occurring or chimeric SSB protein homologous to *Eco*SSB with said DNA and said polymerase

53. The method of claim 52, further comprising translation of said RNA.

54. The method of claim 23, wherein said DNA is single-stranded linear DNA.

55. The method of claim 23, wherein said DNA is single-stranded circular DNA.

56. The method of claim 55, wherein said circular DNA is bacteriophage M13 DNA.

57. The method of claim 23, wherein said DNA is denatured DNA.

58. The method of claim 57, wherein said denatured DNA is single-stranded DNA.

59. The method of claim 57, wherein said denatured DNA is double-stranded linear DNA.

60. The method of claim 57, wherein said denatured DNA is double-stranded circular DNA.

61. The method of claim 23, wherein said RNA is purified RNA.

62. The method of claim 23, wherein said RNA comprises modified nucleotides.

63. The method of claim 23, wherein mixed RNA-DNA oligonucleotides are made.

64. The method of claim 23, wherein no *Eco*SSB is admixed with said RNA polymerase and said DNA and wherein said RNA is in the form of a DNA/RNA hybrid.

65. The method of claim 23, wherein said RNA comprises a detectable label.

66. The method of claim 65, wherein said detectable label is a fluorescent tag.

67. The method of claim 65, wherein said detectable label is biotin.

68. The method of claim 65, wherein said detectable label is digoxigenin.

69. The method of claim 65, wherein said detectable label is 2'-fluoro nucleoside triphosphate.

70. The method of claim 65, wherein said detectable label is a radiolabel.

71. The method of claim 70, wherein said radiolabel is a $^{35}$S- or $^{32}$P-label.

72. The method of claim 65, wherein said RNA is adapted for use as a probe for blotting experiments or in-situ hybridization.

73. The method of claim 23, wherein nucleoside triphosphates (NTPs) are incorporated into said RNA.

74. The method of claim 73, wherein said NTPs comprise a detectable label.

75. The method of claim 75, wherein said NTPs are derivatized NTPs.

76. The method of claim 23, wherein deoxynucleoside triphosphates are incorporated into said RNA.

77. The method of claim 23, wherein said RNA is adapted for NMR structural determination.

78. The method of claim 77, wherein said RNA has between 10 and 1000 bases.

79. The method of claim 78, wherein said RNA has between 10 and 300 bases.

80. The method of claim 23, wherein said RNA is adapted for spliceosome assembly.

81. The method of claim 23, wherein said RNA is adapted for splicing reactions.

82. The method of claim 23, wherein said RNA is adapted for use in antisense experiments.

83. The method of claim 23, wherein said RNA is adapted for use in probing for a complementary nucleotide sequence.

84. The method of claim 23, wherein said RNA is adapted for use as a probe in RNase protection studies.

85. The method of claim 23, further comprising the step of delivering said RNA into a cell.

86. The method of claim 85, wherein delivering is by microinjection.

87. The method of claim 23, further comprising the step of amplifying said RNA.

88. A method of making RNA comprising:

(a) obtaining a N4 virion RNA polymerase;

(b) obtaining a single-stranded DNA oligonucleotide wherein said oligonucleotide contains a N4 virion RNA polymerase promoter sequence;

(c) admixing said RNA polymerase and said oligonucleotide; and

(d) culturing said RNA polymerase and said oligonucleotide under conditions effective to allow RNA synthesis.

89. The method of claim 88, wherein said RNA polymerase has the amino sequence set forth in SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8.

90. The method of claim 88, wherein said DNA has between 20 and 200 bases.

91. A method of making RNA comprising:

(a) obtaining a N4 virion RNA polymerase;

(b) obtaining a single-stranded DNA wherein said DNA contains a N4 virion RNA polymerase promoter sequence;

(c) obtaining a ribonucleoside triphosphate (XTP) or a derivatized ribonucleoside triphosphate;

(d) admixing said RNA polymerase, said DNA and said XTP; and

(e) culturing said RNA polymerase and said oligonucleotide under conditions effective to allow RNA synthesis wherein said RNA is a derivatized RNA.

92. The method of claim 91, wherein said RNA polymerase has the amino sequence set forth in SEQ ID NO:4.

93. The method of claim 91, wherein said RNA polymerase is a mutant of an RNA polymerase comprising the amino sequence essentially as set forth in SEQ ID NO:4 or SEQ ID NO:6.

94. The method of claim 93, wherein said mutant has a mutation at position number Y678.

95. The method of claim 91, wherein said RNA polymerase has the amino sequence set forth in SEQ ID NO:8.

96. A method for *in vivo* protein synthesis comprising:

(a) obtaining an RNA polymerase having the amino sequence set forth in SEQ ID NO:4 or a mutant thereof;

(b) obtaining DNA wherein said DNA contains a N4 virion RNA polymerase promoter sequence;

(c) admixing said RNA polymerase and said DNA;

(d) culturing said RNA polymerase and said DNA under conditions effective to allow RNA synthesis; and

(e) culturing said RNA *in vivo* under conditions effective to allow protein synthesis.

97. The method of claim 96, wherein step (e) comprises using a two plasmid system.

98. The method of claim 96, wherein step (e) comprises using a one plasmid system.

99. The method of claim 98, wherein a reporter gene and said RNA polymerase are on the same plasmid.

100. A method of making a full-length N4 vRNAP or mini-vRNAP comprising:

(a) expressing vRNAP, wherein said vRNAP has the amino sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:15 or a mutant thereof; and

(b) purifying said vRNAP.

101. The method of claim 100, wherein said expressing occurs in a bacteria, yeast, CHO, Cos, HeLa, NIH3T3, Jurkat, 293, Saos, or PC12 host cell.

102. The method of claim 100, further comprising using a promoter appropriate for expression in the host cell line being used.

103. The method of claim 102, wherein said promoter is pBAD.

104. The method of claim 102, wherein said promoter is a promoter recognized by T7 RNA polymerase, T3 RNA polymerase or SP6 RNA polymerase.

105. The method of claim 102, wherein said promoter is a promoter recognized by T7-like RNA polymerase.

106. The method of claim 100, wherein said vRNAP has a specific recombinant sequence for use in purification.

107. The method of claim 106, wherein said vRNAP has at least one histidine, FLAG, hemaglutinin or c-myc tag.

108. The method of claim 106, wherein said vRNAP has at least one histidine tag.

109. The method of claim 107, wherein said purifying occurs in one step.

110. The method of claim 100, wherein said vRNAP does not have a tag.

```
      -11
      A/G
    A     G
     G:C
     C:G
     X:X´
     X:X´          +1
3´   C:G  A  A  A/T  A  C  C   5´
   -17
```

FIG. 1

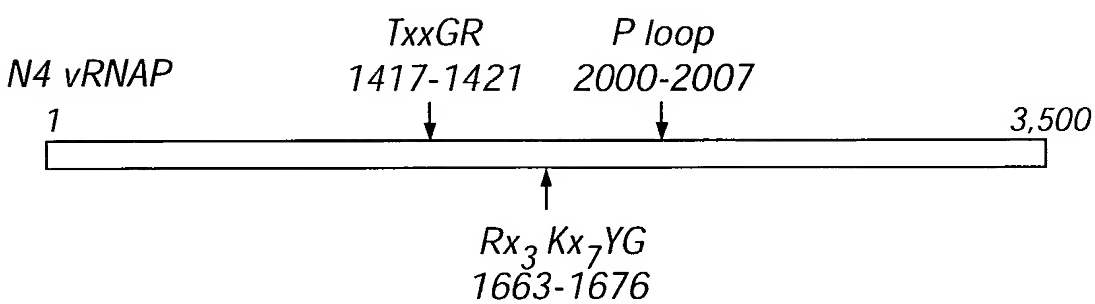



FIG. 2A

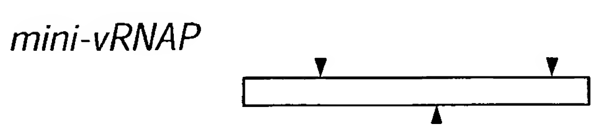



FIG. 2B

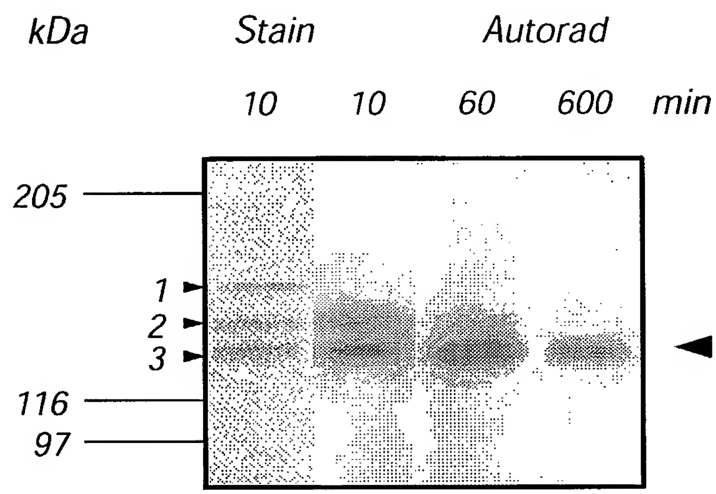

kDa
Stain
Autorad
10
10
60
600
min
205
1
2
3
116
97

FIG. 3A

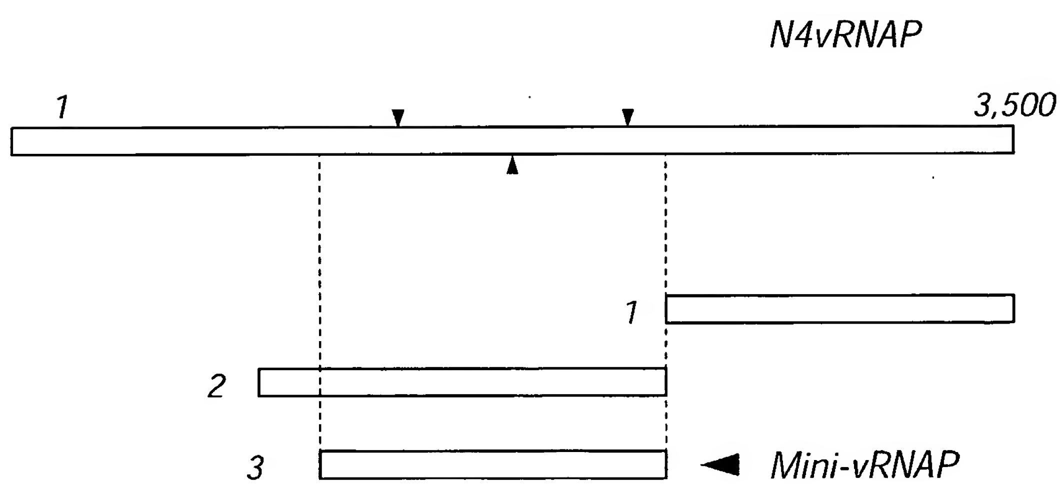

N4vRNAP
1
3,500
1
2
3
Mini-vRNAP

FIG. 3B

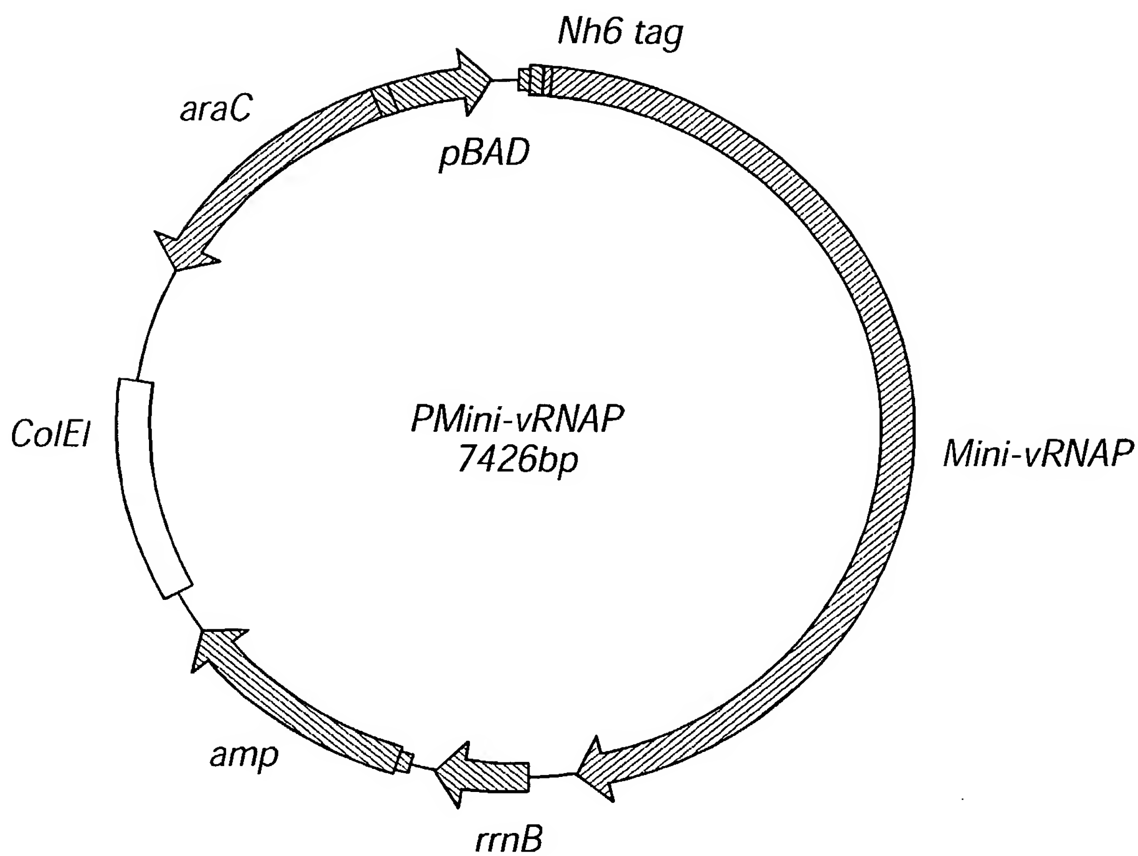

Nh6 tag
araC
pBAD
ColEI
PMini-vRNAP
7426bp
Mini-vRNAP
amp
rrnB

FIG. 4

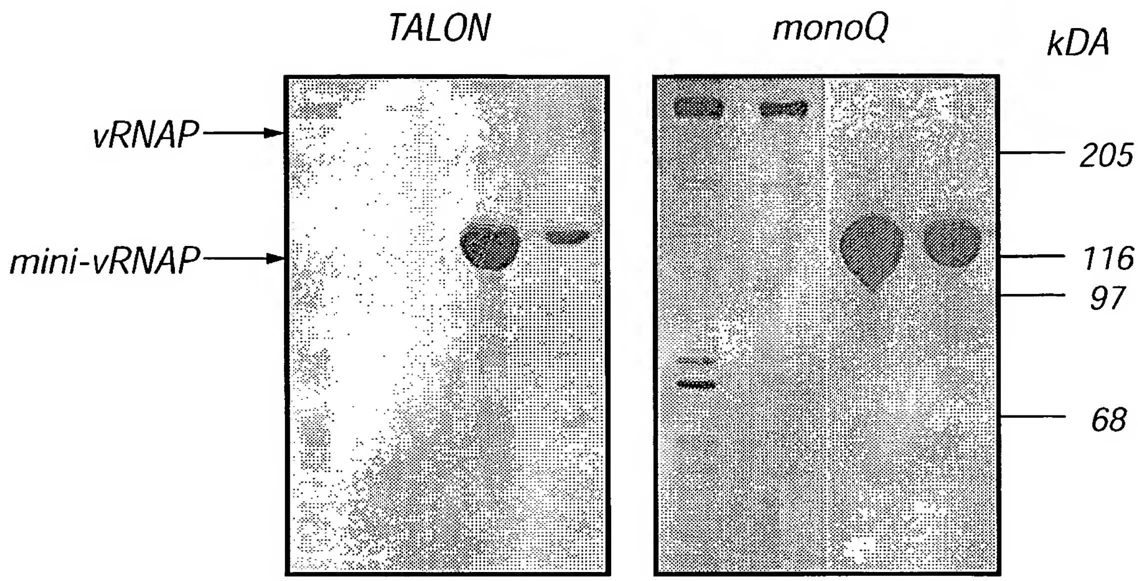

TALON
monoQ
kDA
vRNAP
mini-vRNAP
205
116
97
68

FIG. 5

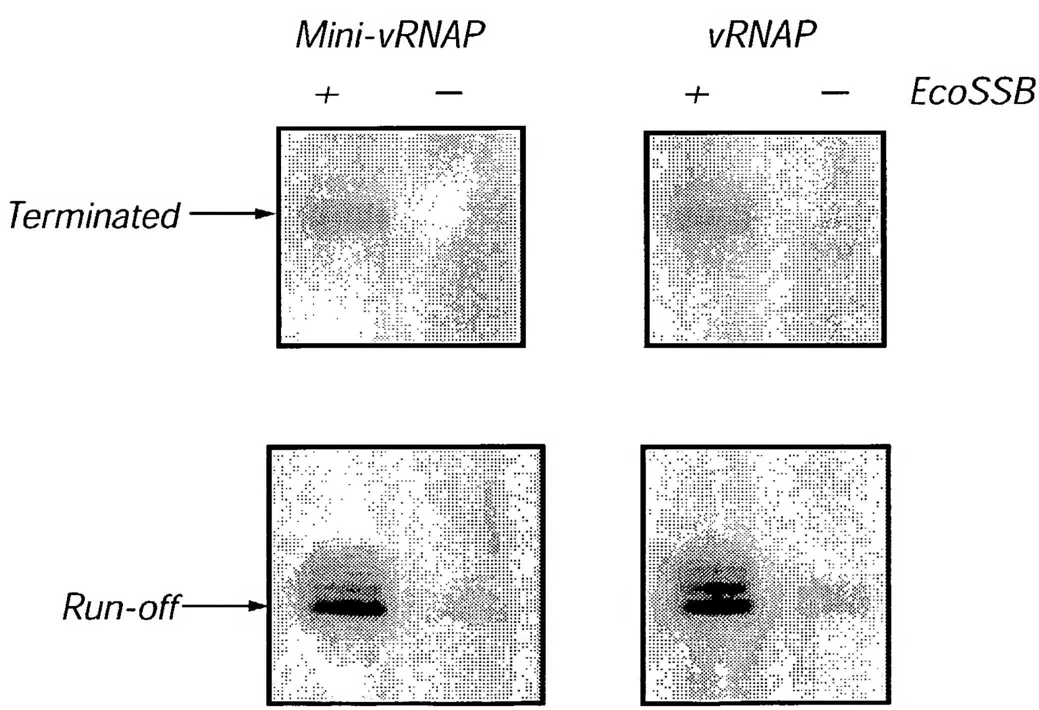

Mini-vRNAP
vRNAP
+
−
+
−
EcoSSB
Terminated
Run-off

FIG. 10

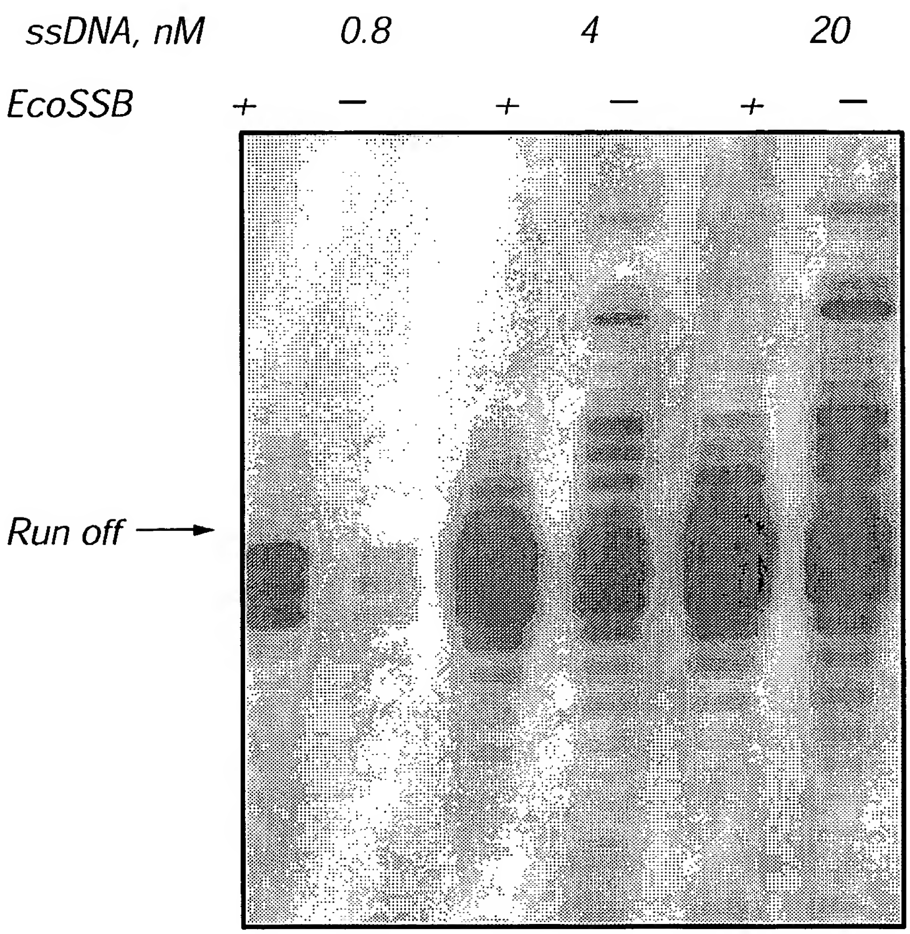



FIG. 6

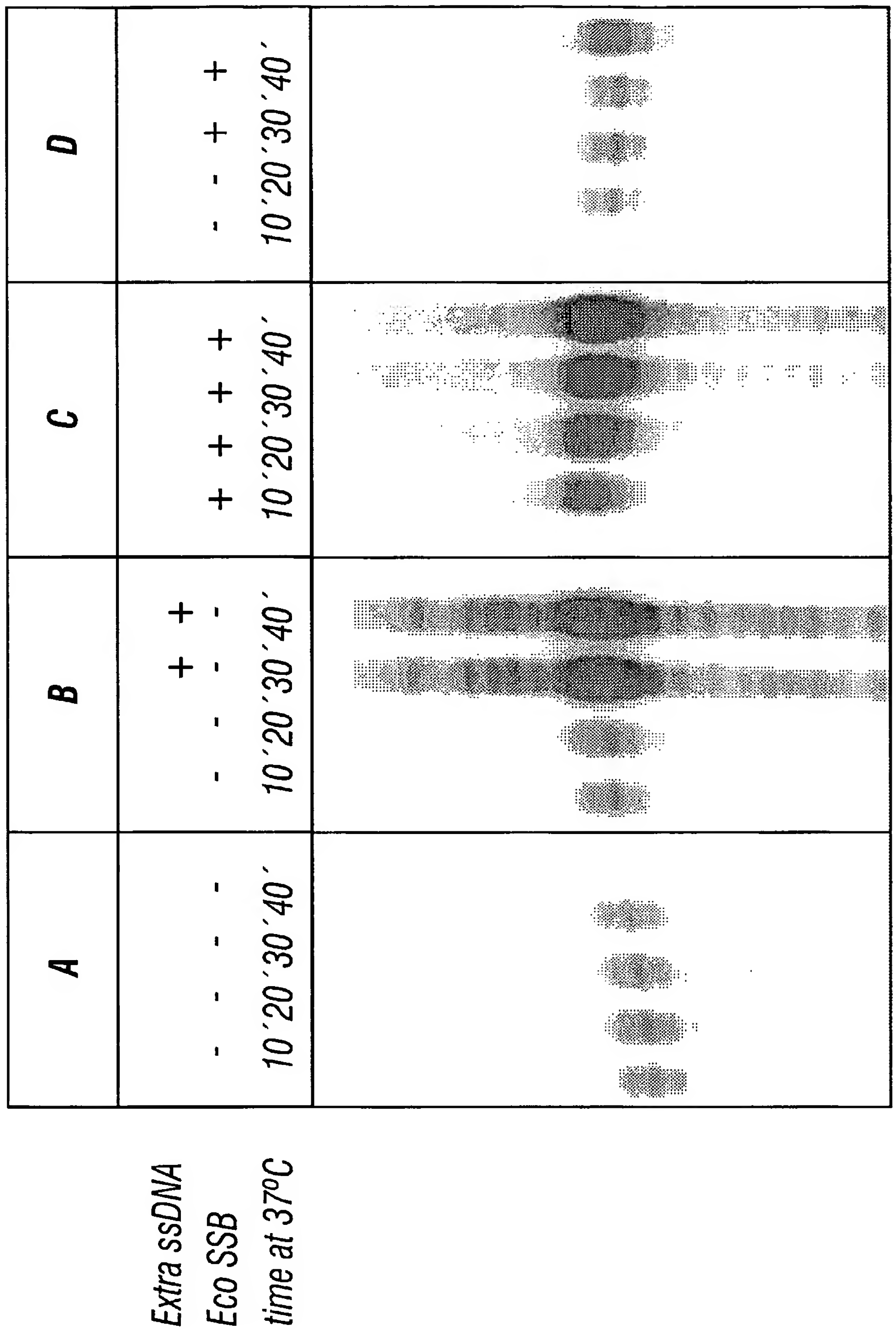
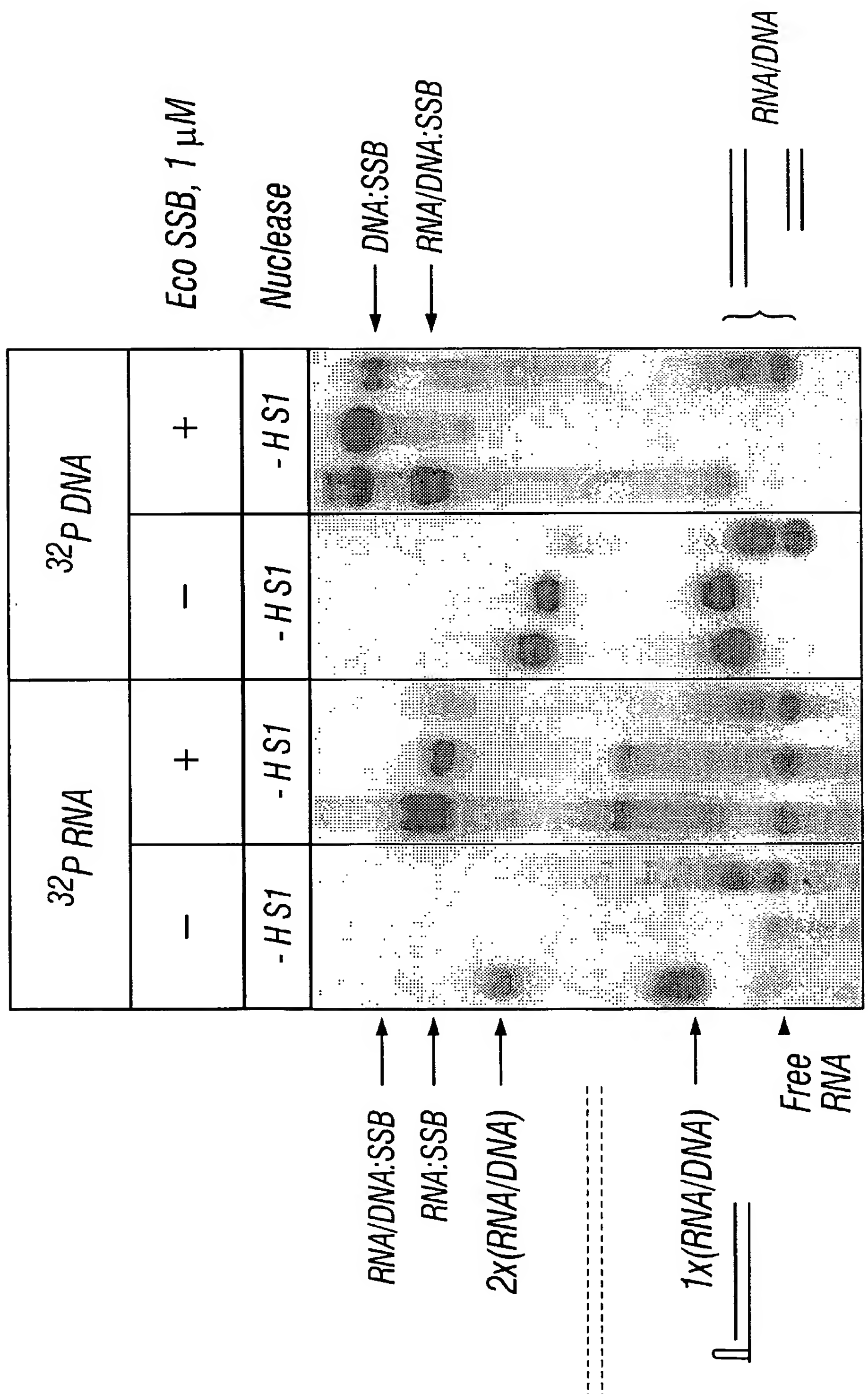



FIG. 7



Eco SSB, 1 μM
Nuclease
DNA:SSB
RNA/DNA:SSB
RNA/DNA
$^{32}P$ DNA
$^{32}P$ RNA
+
−
- H S1
RNA/DNA:SSB
RNA:SSB
2x(RNA/DNA)
1x(RNA/DNA)
Free RNA


FIG. 8

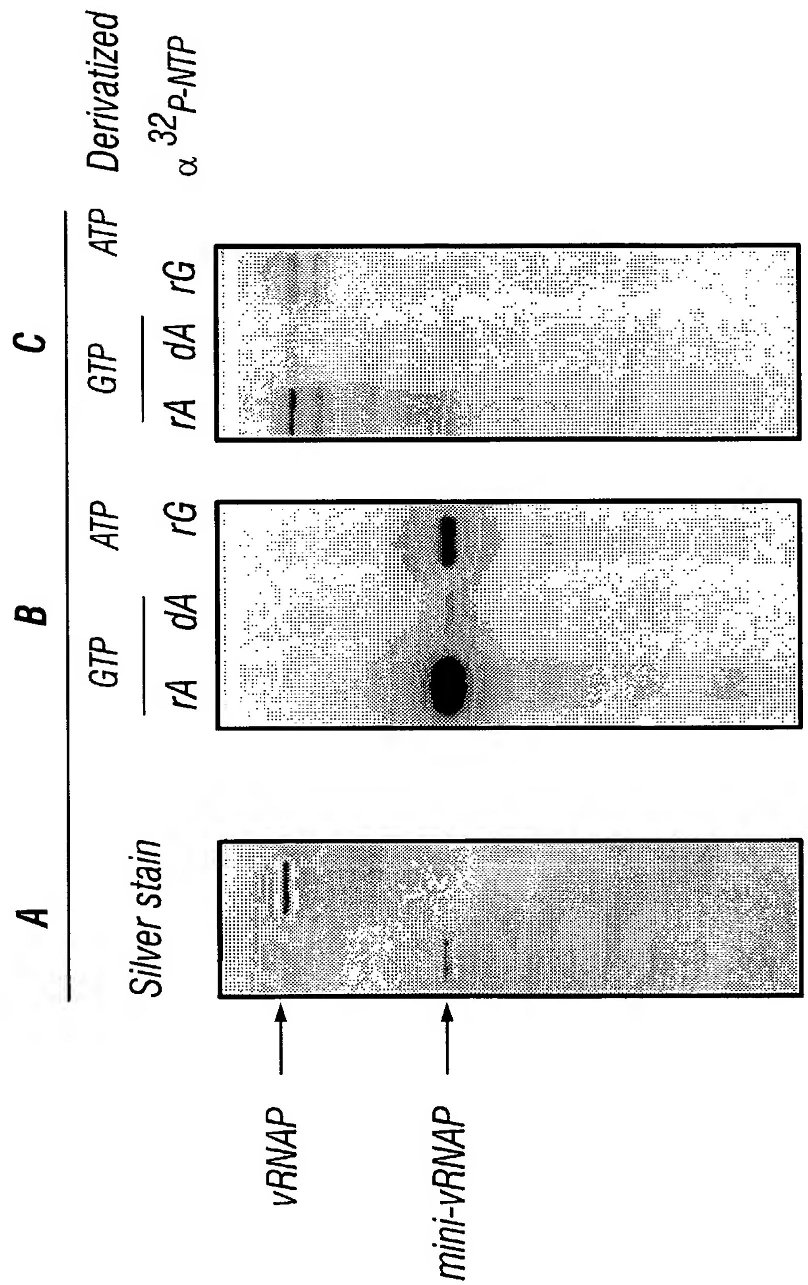

A
Silver stain
B
GTP
ATP
rA
dA
rG
C
GTP
ATP
rA
dA
rG
Derivatized
α $^{32}$P-NTP
vRNAP
mini-vRNAP

FIG. 9

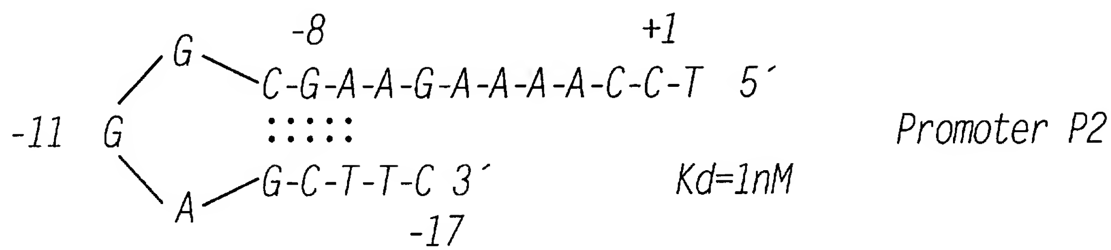



FIG. 11A

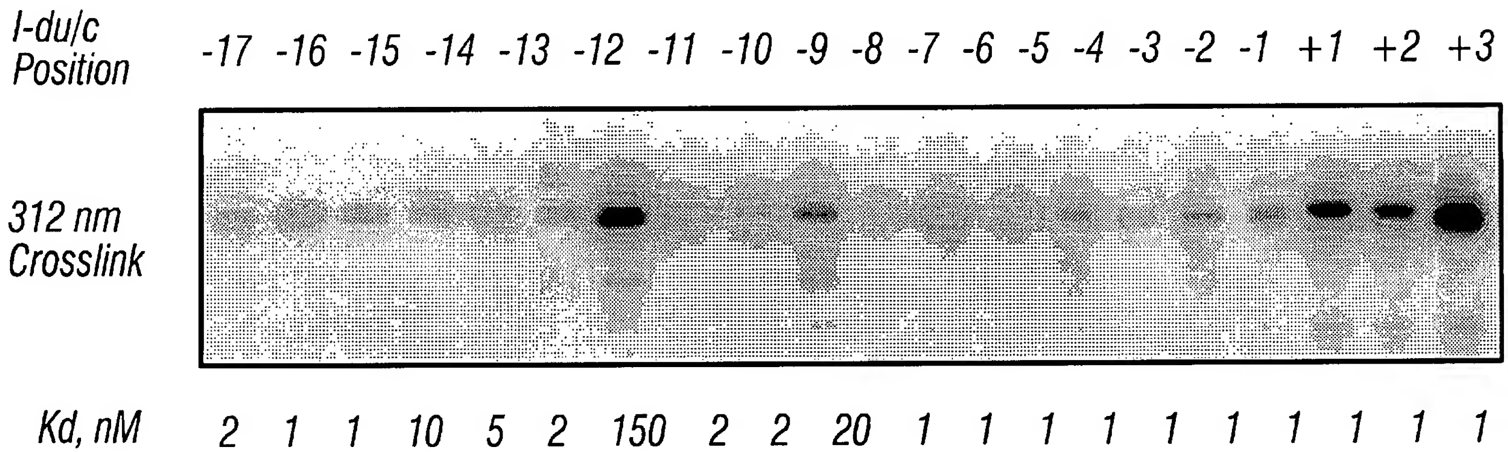



FIG. 11B

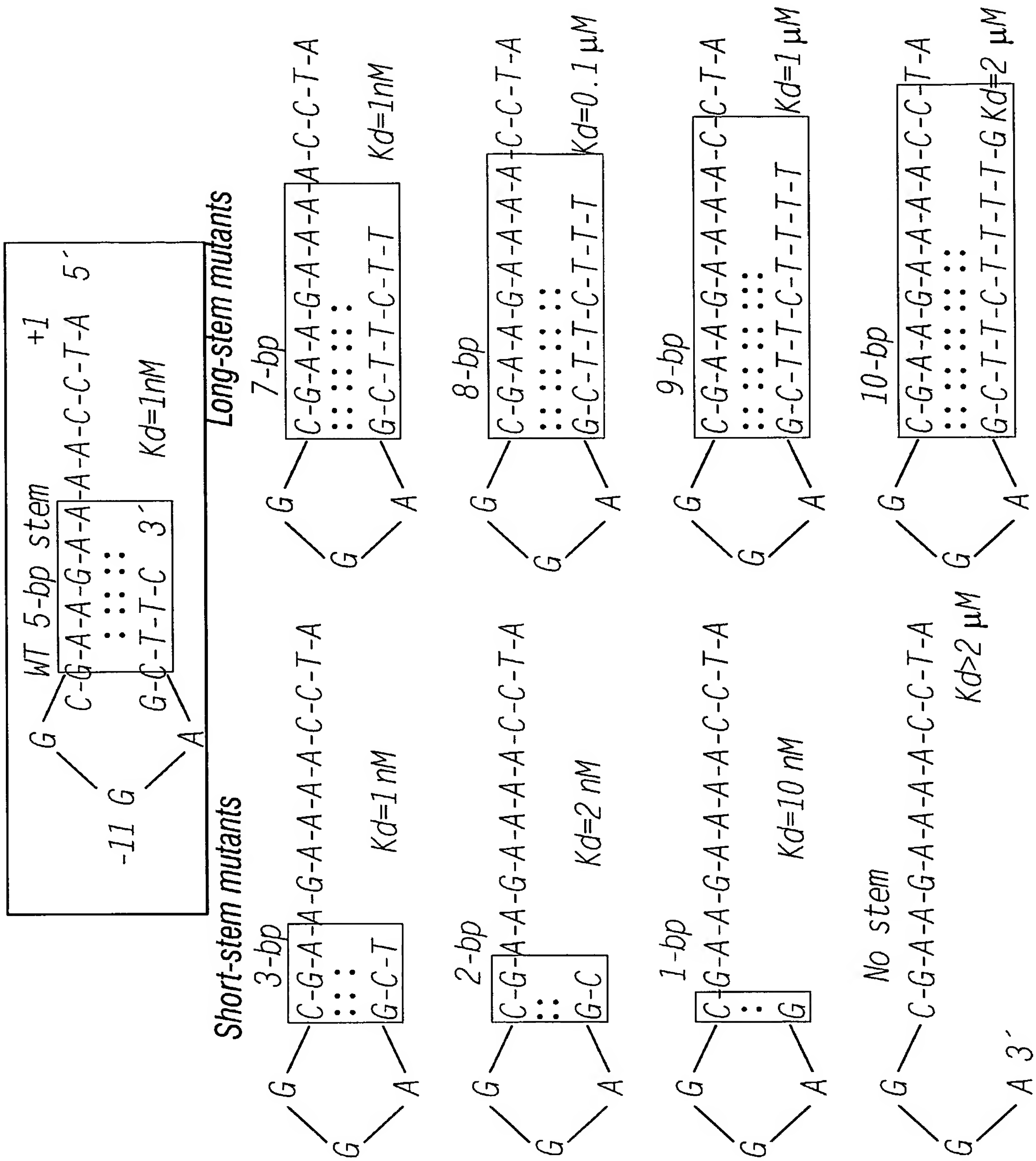

WT 5-bp stem
+1
5´
3´
-11 G
Kd=1nM
Long-stem mutants
7-bp
Kd=1nM
8-bp
Kd=0.1 µM
9-bp
Kd=1 µM
10-bp
Kd=2 µM
Short-stem mutants
3-bp
Kd=1 nM
2-bp
Kd=2 nM
1-bp
Kd=10 nM
No stem
Kd>2 µM
A 3´

FIG. 12

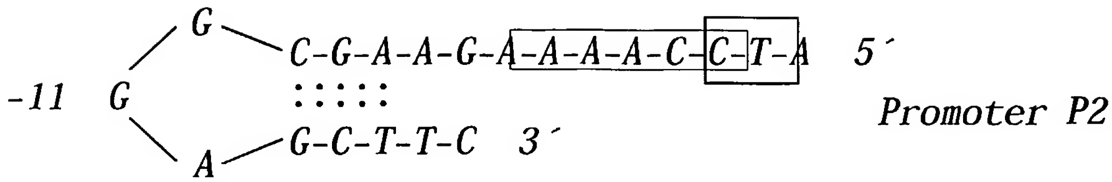



**FIG. 13A**

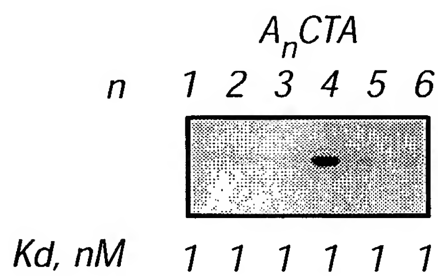



**FIG. 13B**

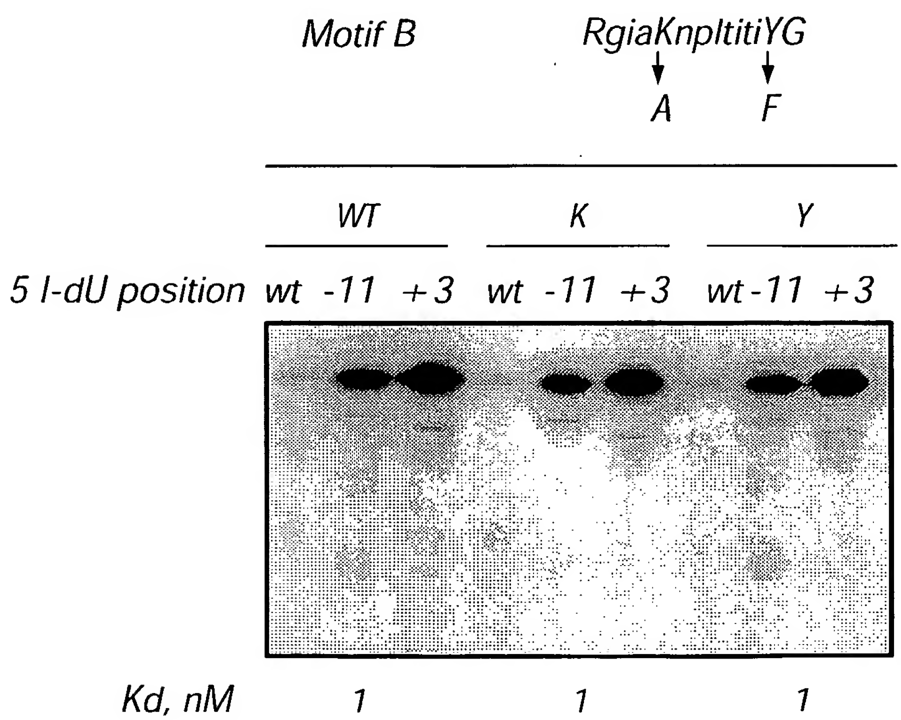



**FIG. 14**

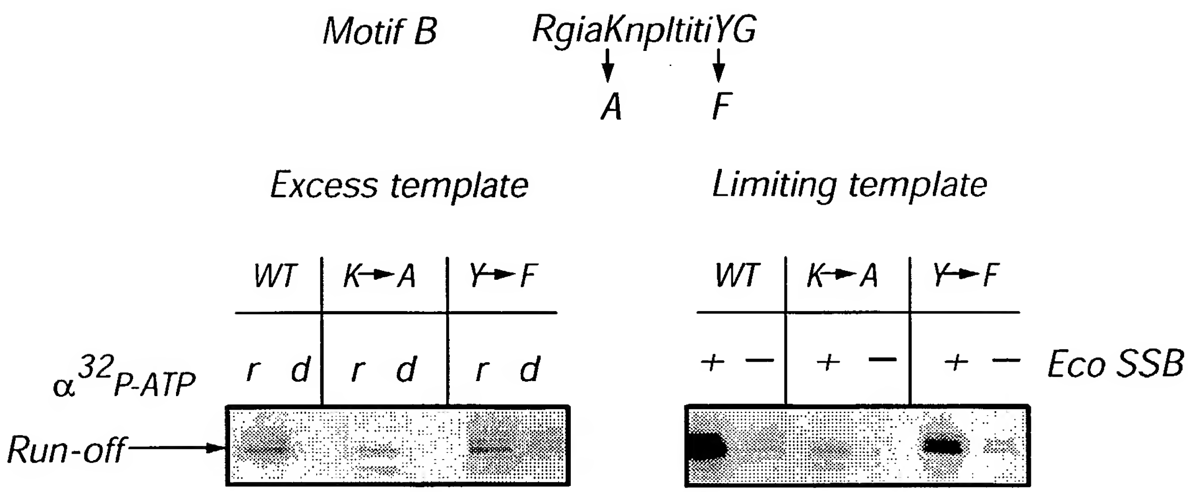

Motif B
RgiaKnpltitiYG
A
F
Excess template
Limiting template
WT
K→A
Y→F
$\alpha^{32}$P-ATP
r d r d r d
+ − + − + −
Eco SSB
Run-off

FIG. 15

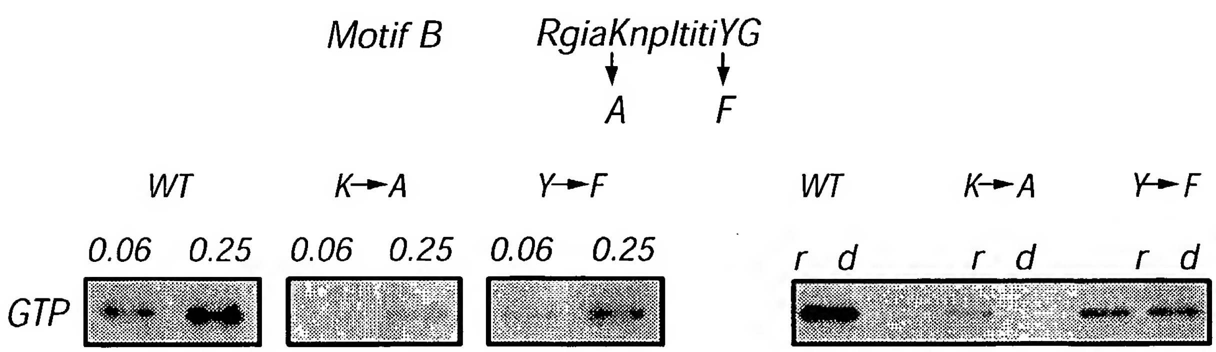

Motif B
RgiaKnpltitiYG
A
F
WT
K→A
Y→F
0.06 0.25 0.06 0.25 0.06 0.25
r d r d r d
GTP

FIG. 16

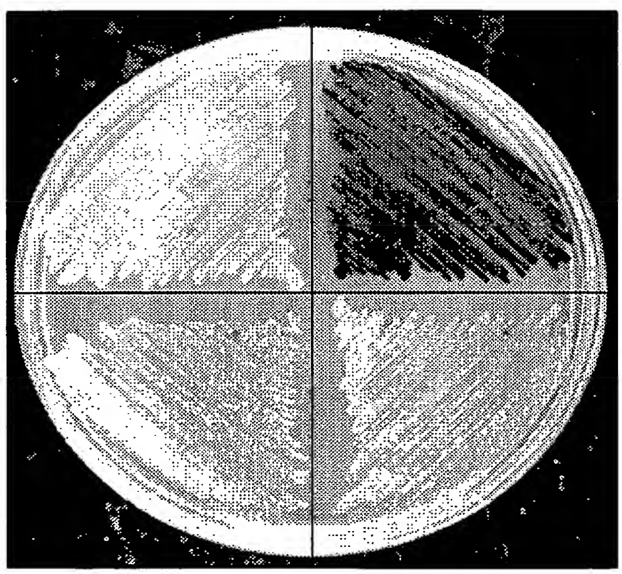



FIG. 17A

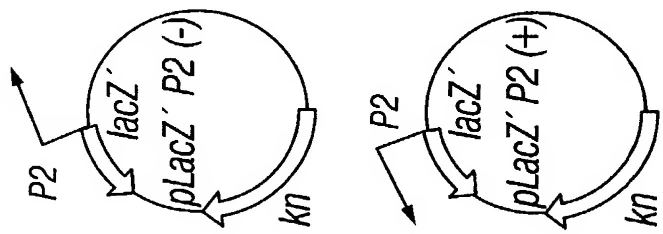



FIG. 17B

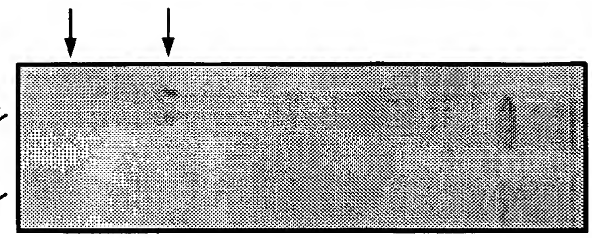



FIG. 17C

SEQUENCE LISTING

<110> KAZMIERCZAK, KRYSTYNA M.
DAVYDOVA K.
ROTHMAN-DENES B.

<120> N4 VIRION SINGLE STRANDED DNA DEPENDENT RNA POLYMERASE

<130> ARCD:375-WO

<140> UNKNOWN
<141> 2002-05-22

<150> 60/292,845
<151> 2001-05-22

<160> 29

<170> PatentIn Ver. 2.1

<210> 1
<211> 10506
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 1
atgtcagtat ttgatagact ggctgggttc gcagacagcg taaccaatgc aaagcaagtt 60
gacgtctcta ctgcaaccgc ccagaagaaa gctgaacaag gtgtcactac tcctcttgtt 120
tctcctgatg ctgcttatca aatgcaagct gcccgtactg gtaatgttgg ggctaatgca 180
tttgaaccag ggacagtgca atcagatttc atgaatctga ccccaatgca aatcatgaat 240
aagtatgggg ttgagcaagg cttacaactt atcaatgctc gtgctgatgc agggaaccag 300
gtattcaatg attcagttac tacaagaact cctggggaag aactggggga tattgctact 360
ggtgttggcc ttggttttgt taataccctt gggggcattg gtgctcttgg ggcaggctta 420
ctcaacgatg atgcaggtgc tgttgttgct caacaattga gtaagtttaa tgatgctgtt 480
catgctaccc aaagccaggc attacaagat aaacgtaagc tctttgctgc tcgtaactta 540
atgaatgaag tagagagtga acgtcagtat caaacagata agaaagaagg cactaatgac 600
atagtagctt ccttatctaa atttggacgt gattttgtag gttcaattga gaatgctgct 660
caaactgact ctattatttc tgatgggtta gcagaagggg taggttctct attaggtgct 720
ggtcctgtat taaggggtgc atctttactg ggtaaagcag ttgttccagc aaatactctt 780
cgtagtgctg cattggctgg tgctattgat gcaggtactg gtactcagtc actggctcgt 840
attgcctcta ctgtaggtag agctgcaccg ggtatggttg gtgttggtgc aatggaagct 900
ggtggtgcat accaacaaac tgctgatgaa attatgaaga tgagtcttaa agacttagag 960
aagtctcctg tttatcagca acatattaaa gatggtatgt cccctgaaca ggctcgtcgt 1020
cagactgcat ctgaaactgg tcttactgct gctgctattc aattacctat tgctgctgca 1080
accggtcctc tggtatcccg ttttgagatg gctcctttcc gtgctggctc tttaggtgct 1140
gtaggtatga accttgcccg tgaaacagtg gaagaaggtg ttcagggtgc tacaggccaa 1200
ctggctcaga atattgcaca gcaacaaaac attgataaga accaagacct gcttaaaggt 1260
gtcggtacac aggctggttt aggtgctctt tatggctttg gttctgctgg tgttgtacag 1320
gctccggctg gtgctgctcg tttagcaggt gctgcaactg ctcctgtatt gcgtaccaca 1380
atggctggtg ttaaagctgc tggtagtgta gcaggtaagg ttgtttctcc tattaagaat 1440
actttagtag ctcgtggtga acgggttatg aagcagaatg aagaagcatc tcctgttgct 1500
gatgactatg ttgcacaggc agcacaagaa gctatggctc aagcaccaga agcagaagtt 1560
actattcgtg atgctgttga agcaactgat gctactccag aacagaaagt tgcagcacac 1620
cagtatgttt ctgacttaat gaatgctact cgttttaatc ctgaaaatta tcaggaagca 1680
ccagagcata ttcgtaatgc tgtagctggt tctactgacc aagtacaggt tattcagaag 1740
ttagcagact tagttaacac attagatgaa tctaatcctc aagcactgat ggaagctgca 1800

```
tcttatatgt atgatgctgt ttcagagttt gagcagttca ttaaccgtga ccctgctgca 1860
ctggatagca ttcctaaaga ttctccggct attgagttac tcaaccgtta tacgaatctg 1920
acagctaata ttcagaacac accaaaagta attggtgcac tgaatgttat taatcgaatg 1980
attaatgaat ctgctcagaa tggttctttg aatgtgactg aagaatccag tccacaggaa 2040
atgcagaacg tagcattagc tgctgaagta gcccctgaaa agctcaatcc agagtctgta 2100
aatgttgttc ttaaacatgc tgctgatggt cgtattaaac tgaataatcg ccagattgct 2160
gccctccaga atgctgctgc aatcctgaag gggcacgggg aatatgatgc agaagctgcc 2220
cgtcttggat tacgtcctca agacattgtg agtaaacaga ttaaaacgga tgagagcaga 2280
actcaggaag gacaatactc tgcgttgcaa catgcgaata ggattcggtc tgcgtataac 2340
tctggtaatt tcgagttggc ctccgcttac ctgaacgact ttatgcagtt cgcccagcac 2400
atgcagaata aggttggagc gttgaatgag catcttgtta cggggaatgc ggataagaat 2460
aagtctgtcc actaccaagc tcttactgct gacagagaat gggttcgtag ccgtaccgga 2520
ttgggggtca atccctatga cactaagtcg gttaaatttg cccagcaagt tgctcttgaa 2580
gcgaaaacgg tagcggatat tgctaatgcc ctcgcttcgg cttacccgga actgaaggtc 2640
agtcatataa aagttactcc attggattca cgtcttaacg ctcctgctgc tgaggtggtc 2700
aaggcattcc gtcaaggcaa tcgagacgtt gcttcttctc aaccgaaagc tgactccgtg 2760
aatcaggtta aagaaactcc tgttacaaaa caggaaccag ttacatctac tgtacagact 2820
aagactcctg ttagtgaatc tgttaaaaca gaacctacta ctaaagagtc tagcccacag 2880
gctataaaag aacctgtgaa ccagtctgaa aaacaggatg ttaacccttac taatgaggac 2940
aacatcaagc aacctactga atctgttaaa gaaactgaaa cttctacaaa agaaagtaca 3000
gttacagaag aattaaaaga aggtattgat gctgtttacc cttcattggt aggtactgct 3060
gattctaaag cagagggtat taagaactat ttcaaattgt cctttacctt accagaagaa 3120
cagaaatccc gtactgttgg ttcagaagca cctctaaaag atgtagccca agctctgtct 3180
tctcgtgctc gttatgaact ctttactgag aaagaaactg ctaaccctgc ttttaatggg 3240
gaagttatta agcgatacaa agaactcatg gaacatgggg aaggtattgc tgatattctt 3300
cgctcccgtc tggctaagtt ccttaacact aaggatgttg gtaaacgttt tgctcaaggt 3360
acagaagcca accgttgggt aggtggtaag ttacttaaca ttgttgagca ggatggggat 3420
acctttaagt acaacgaaca attgctacag actgctgtat tagcaggtct tcaatggaga 3480
cttactgcta ccagcaatac tgctatcaaa gatgcaaaag atgttgctgc tattactggt 3540
attgaccaag ctctgctgcc agaaggttta gtagagcaat ttgatactgg tatgacactc 3600
actgaagcag ttagttccct ggctcagaaa attgagtctt actggggatt atctcgtaat 3660
ccaaatgctc cattgggcta taccaaaggc atccctacag caatggctgc tgaaattctg 3720
gctgcatttg tagagtctac tgatgttgta gagaacatcg tggatatgtc agaaattgac 3780
ccagataaca agaagactat tggtctgtac accattactg aactggattc cttcgaccca 3840
attaatagct tccctactgc tattgaagaa gctgttttag tgaatcctac agagaagatg 3900
ttctttggtg atgacattcc tcctgtagct aatactcagc ttcgtaaccc tgctgttcgt 3960
aatactccag aacagaaggc tgcattgaaa gcagagcagg ctacagagtt ctatgtacac 4020
accccaatgg ttcaattcta tgagacgtta ggtaaagacc gtattctcga actgatgggt 4080
gctggtactc tgaataaaga gttacttaat gataaccatg ctaaatctct ggaaggtaag 4140
aaccgttcag tagaggactc ttacaaccaa ctgttctccg tcattgagca ggtaagagca 4200
cagagcgaag acatctctac tgtacctatt cactatgcat acaatatgac ccgtgttggt 4260
cgtatgcaga tgttaggtaa atacaatcct caatcagcca aactggttcg tgaggccatc 4320
ttacctacta aagctacttt ggatttatcg aaccagaaca atgaagactt ctctgcattc 4380
cagttaggtc tggctcaggc attggacatt aaagtccata ctatgactcg tgaggttatg 4440
tctgacgagt tgactaaatt actggaaggt aatctgaaac cagccattga tatgatggtt 4500
gagtttaata ccactggttc cttaccagaa aacgcagttg atgttctgaa tacagcatta 4560
ggagatagga agtcattcgt agcattgatg gctcttatgg agtattcccg ttacttagta 4620
gcagaggata aatctgcatt tgtaactcca ctgtatgtag aagcagatgg tgttactaat 4680
ggtccaatca atgccatgat gctaatgaca ggcggtctgt ttactcctga ctggattcgt 4740
aatattgcca aagggggctt gttcattggt tctccaaata agaccatgaa tgagcatcgc 4800
tctactgctg acaataatga tttatatcaa gcatccacta atgctttgat ggaatcgttg 4860
ggtaagttac gtagtaacta tgcctctaat atgcctattc agtctcagat agacagtctt 4920
ctttctctga tggatttgtt tttaccggat attaatcttg gtgagaatgg tgctttagaa 4980
cttaaacgtg gtattgctaa gaacccactg actattacca tctatggttc tggtgctcgt 5040
ggtattgcag gtaagctggt tagttctgtt actgatgcca tctatgagcg tatgtctgat 5100
gtactgaaag ctcgtgctaa agacccaaat atctctgctg ctatggcaat gtttggtaag 5160
caagctgctt cagaagcaca tgctgaagaa cttcttgccc gtttcctgaa agatatggaa 5220
acactgactt ctactgttcc tgttaaacgt aaaggtgtac tggaactaca atccacaggt 5280
acaggagcca aaggaaaaat caatcctaag acctatacca ttaagggcga gcaactgaag 5340
gcacttcagg aaaatatgct gcacttcttt gtagaaccac tacgtaatgg tattactcag 5400
```

actgtaggtg aaagtctggt gtactctact gaacaattac agaaagctac tcagattcaa 5460
tctgtagtgc tggaagatat gttcaaacag cgagtacaag agaagctggc agagaaggct 5520
aaagacccaa catggaagaa aggtgatttc cttactcaga aagaactgaa tgatattcag 5580
gcttctctga ataacttagc ccctatgatt gagactggtt ctcagacttt ctacattgct 5640
ggttcagaaa atgcagaagt agcaaatcag gtattagcta ctaaccttga tgaccgtatg 5700
cgtgtaccaa tgagtatcta tgctccagca caggccggtg tagcaggtat tccatttatg 5760
actattggta ctggtgatgg catgatgatg caaactcttt ccactatgaa aggtgcacca 5820
aagaataccc tcaaaatctt tgatggtatg aacattggtt tgaatgacat cactgatgcc 5880
agtcgtaaag ctaatgaagc tgtttacact tcttggcagg gtaaccctat taagaatgtt 5940
tatgaatcat atgctaagtt catgaagaat gtagatttca gcaagctgtc ccctgaagca 6000
ttggaagcaa ttggtaaatc tgctctggaa tatgaccaac gtgagaatgc tactgtagat 6060
gatattgcta acgctgcatc tctgattgaa cgtaacttac gtaatattgc actgggtgta 6120
gatattcgtc ataaggtgct ggataaggta aatctgtcca ttgaccagat ggctgctgta 6180
ggtgctcctt atcagaacaa cggtaagatt gacctcagca atatgacccc tgaacaacag 6240
gctgatgaac tgaataaact tttccgtgaa gagttagaag cccgtaaaca aaaagtcgct 6300
aaggctaggg ctgaagtcaa agaagaaact gtttctgaaa aagaaccagt gaatccagac 6360
tttggtatgg taggccgtga gcataaggca tctggtgttc gtatcctgtc tgctactgct 6420
attcgtaatc tggctaagat tagtaatctg ccatctactc aggcagctac tcttgcggag 6480
attcagaaat cactggcagc taaagactat aagattatct acggtacacc tactcaggtt 6540
gcagagtatg ctcgtcagaa gaatgttact gaattgactt ctcaggaaat ggaagaagct 6600
caggcaggta atatttatgg ctggactaac ttcgatgata agaccattta tctggttagc 6660
ccatctatgg aaaccctcat tcatgaactg gttcatgcct ctaccttcga ggaagtttat 6720
tccttctatc agggtaatga agtaagccct acttctaagc aggctattga gaaccttgaa 6780
ggtctgatgg aacagttccg ttctctggat atttccaaag attctccaga aatgagagaa 6840
gcatatgctg atgctattgc aactatcgaa ggtcatttga gtaatggatt tgttgaccca 6900
gctatctcta aagctgctgc tcttaatgag tttatggctt gggggttagc taaccgtgct 6960
cttgctgcta aacagaagag aacatcttca ctggttcaaa tggtgaaaga tgtttatcag 7020
gctattaaga aattgatttg ggacgtaaaa caagctcctg cattgggaga agatatgttc 7080
tccaatctgc tgtttaactc tgcaattctg atgcgtagcc aacctacaac tcaggcagta 7140
gctaaagatg gcacactgtt ccatagcaaa gcatatggta ataatgaacg tctgtctcag 7200
ttgaaccaga ctttcgataa actggtaact gattaccttc gtactgaccc agttacagaa 7260
gtagaacgtc gtggcaatgt ggctaatgca ttaatgagtg ctactcgact ggttcgtgat 7320
gttcagtctc atggcttcaa tatgactgct caggaacagt ctgtattcca gatggttact 7380
gctgcattag caactgaagc tgcgattgac ccacatgcta tggctcgtgc tcaggaactt 7440
tatacccatg taatgaaaca ccttacggta gagcatttca tggctgaccc tgatagtact 7500
aaccctgctg accgttacta tgctcaacag aaatatgaca ccatctctgg tgctaatctg 7560
gttgaagtag atgccaaagg tagaaccagt ctgttaccta cattcctggg tctggctatg 7620
gttaatgaag aactacgttc aatcattaaa gaaatgcctg tacctaaagc agataagaaa 7680
ttagggaatg atatagatac tctgcttacc aatgcaggta ctcaggtaat ggaatctctg 7740
aaccgtcgta tggctggtga ccagaaagct actaatgttc aggacagtat tgatgctttg 7800
tcagaaacaa tcatggctgc tgctttgaaa cgagagtcct tctatgatgc tgtagcaacc 7860
cctaccggta acttcattga ccgtgctaat cagtacgtaa cggatagcat tgaacggtta 7920
tctgaaactg ttattgagaa ggcagataag gtaattgcta acccttctaa tatagctgct 7980
aaaggtgttg ctcatctggc taaactgact gctgctattg catctgaaaa acagggtgaa 8040
atagtggctc agggtgttat gactgctatg aaccagggta aagtatggca acctttccat 8100
gacttagtta atgacattgt tggccgtact aagactaatg ccaatgtcta tgacttaatc 8160
aaattggtta agagccagat ttctcaagac cgtcagcaat tccgtgagca tttacctaca 8220
gtcattgctg gtaagttctc tcgtaaattg actgataccg aatggtctgc aatgcatact 8280
ggtttaggta aaacagattt agctgttcta cgtgaaacta tgagcatggc tgaaattaga 8340
gatttactct cttcatccaa gaaagtgaaa gatgaaatct ctactctgga aaaagagatt 8400
cagaaccaag caggtagaaa ctggaatctg gttcagaaga aatctaagca actggctcaa 8460
tacatgatta tgggggaagt aggtaataac ctccttcgta atgcccatgc tattagtcgt 8520
ttgttaggtg aacgtattac taatggtcct gtggcagatg tagctgctat tgataagctc 8580
attactttgt actctctgga attgatgaat aagtctgacc gtgacctttt gtcagaattg 8640
gctcaatcag aagtggaagg tatggagttc tccattgctt atatggttgg tcaacgtact 8700
gaagagatgc gtaaagctaa aggtgataac cgtactctgc tgaatcactt taaaggctat 8760
atccctgtag agaaccagca aggtgtgaat ttgattattg ctgacgataa agagtttgct 8820
aagttaaata gccaatcctt tactcgtatt ggtacttatc aggggagcac tggtttccgt 8880
actggttcta aaggttatta cttcagccca gtagctgccc gtgcccctta ctctcagggt 8940
attcttcaga acgttcgtaa tactgctggt ggtgtggata ttggtactgg ctttacgtta 9000

ggcactatgg ttgctgggcg tattactgac aaaccaaccg tagagcgtat taccaaagct 9060
ctggctaaag gtgagcgtgg gcgtgaacca ctgatgccaa tttataacag caaaggtcag 9120
gtagttgctt atgaacaatc cgttgaccct aatatgttga agcacctaaa ccaagacaat 9180
cactttgcta agatggttgg tgtatggcgt ggtcgtcagg tggaagaggc taaagcacaa 9240
cgttttaatg acattctcat tgagcaatta catgctatgt atgagaaaga cattaaagac 9300
tccagtgcta ataaatctca atatgtaaac ctgttaggta aaattgatga cccagtactg 9360
gctgatgcga ttaacctgat gaacattgag actcgtcata aggccgaaga actcttcggt 9420
aaagatgagt tatgggttcg tagggatatg ctgaatgatg cacttggcta tcgtgctgca 9480
tctattggtg atgtgtggac cggtaactct cgttggtcac ctagcaccct tgatactgtt 9540
aagaagatgt tcctcggtgc attcggtaat aaggcatatc atgtagtaat gaatgctgaa 9600
aataccattc agaacttagt gaaggacgct aagacagtaa ttgttgttaa atctgttgta 9660
gtaccggcag ttaacttcct tgctaacatc taccagatga ttggacgtgg tgttcctgtt 9720
aaagatattg ctgtgaacat tcctcgtaag acgtcagaga ttaatcagta tattaaatct 9780
cgtttacgtc agattgatgc ggaagcagag ctacgtgctg ctgaaggtaa ccctaatctg 9840
gttcgtaaac ttaaaactga gattcaatct attactgata gtcatcgtcg tatgagtatc 9900
tggcctttga ttgaagcagg tgagttctct tctattgctg atgctggtat tagtcgtgat 9960
gacctgttag tagctgaagg taagattcat gagtacatgg aaaaacttgc taataaactt 10020
ccagaaaaag tacgtaatgc tggccgttac gctcttattg ctaaggacac tgctctgttc 10080
cagggtatcc agaaaacagt agagtattca gactttattg ctaaagccat catctatgat 10140
gatttagtga aacgtaagaa aaaatcttct tctgaagcat taggtcaggt aactgaagag 10200
tttattaact atgacagatt gcctggtcgt ttccgtggct atatggaaag tatgggtctg 10260
atgtggttct acaactttaa aattcgttcc attaaagttg ctatgagcat gattagaaac 10320
aacccagtac attctctgat tgctacagta gtacctgctc ctaccatgtt tggtaacgta 10380
ggtctaccaa ttcaggacaa catgctaacc atgctggctg aaggaagact ggattactca 10440
ttaggcttcg gacaaggatt aagagcacct accctcaatc cttggttcaa ccttactcac 10500
taataa 10506

<210> 2
<211> 3500
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 2
Met Ser Val Phe Asp Arg Leu Ala Gly Phe Ala Asp Ser Val Thr Asn
1 5 10 15

Ala Lys Gln Val Asp Val Ser Thr Ala Thr Ala Gln Lys Lys Ala Glu
20 25 30

Gln Gly Val Thr Thr Pro Leu Val Ser Pro Asp Ala Ala Tyr Gln Met
35 40 45

Gln Ala Ala Arg Thr Gly Asn Val Gly Ala Asn Ala Phe Glu Pro Gly
50 55 60

Thr Val Gln Ser Asp Phe Met Asn Leu Thr Pro Met Gln Ile Met Asn
65 70 75 80

Lys Tyr Gly Val Glu Gln Gly Leu Gln Leu Ile Asn Ala Arg Ala Asp
85 90 95

Ala Gly Asn Gln Val Phe Asn Asp Ser Val Thr Thr Arg Thr Pro Gly
100 105 110

Glu Glu Leu Gly Asp Ile Ala Thr Gly Val Gly Leu Gly Phe Val Asn

115 120 125

Thr Leu Gly Gly Ile Gly Ala Leu Gly Ala Gly Leu Leu Asn Asp Asp
130 135 140

Ala Gly Ala Val Val Ala Gln Gln Leu Ser Lys Phe Asn Asp Ala Val
145 150 155 160

His Ala Thr Gln Ser Gln Ala Leu Gln Asp Lys Arg Lys Leu Phe Ala
165 170 175

Ala Arg Asn Leu Met Asn Glu Val Glu Ser Glu Arg Gln Tyr Gln Thr
180 185 190

Asp Lys Lys Glu Gly Thr Asn Asp Ile Val Ala Ser Leu Ser Lys Phe
195 200 205

Gly Arg Asp Phe Val Gly Ser Ile Glu Asn Ala Ala Gln Thr Asp Ser
210 215 220

Ile Ile Ser Asp Gly Leu Ala Glu Gly Val Gly Ser Leu Leu Gly Ala
225 230 235 240

Gly Pro Val Leu Arg Gly Ala Ser Leu Leu Gly Lys Ala Val Val Pro
245 250 255

Ala Asn Thr Leu Arg Ser Ala Ala Leu Ala Gly Ala Ile Asp Ala Gly
260 265 270

Thr Gly Thr Gln Ser Leu Ala Arg Ile Ala Ser Thr Val Gly Arg Ala
275 280 285

Ala Pro Gly Met Val Gly Val Gly Ala Met Glu Ala Gly Gly Ala Tyr
290 295 300

Gln Gln Thr Ala Asp Glu Ile Met Lys Met Ser Leu Lys Asp Leu Glu
305 310 315 320

Lys Ser Pro Val Tyr Gln Gln His Ile Lys Asp Gly Met Ser Pro Glu
325 330 335

Gln Ala Arg Arg Gln Thr Ala Ser Glu Thr Gly Leu Thr Ala Ala Ala
340 345 350

Ile Gln Leu Pro Ile Ala Ala Ala Thr Gly Pro Leu Val Ser Arg Phe
355 360 365

Glu Met Ala Pro Phe Arg Ala Gly Ser Leu Gly Ala Val Gly Met Asn
370 375 380

Leu Ala Arg Glu Thr Val Glu Glu Gly Val Gln Gly Ala Thr Gly Gln
385 390 395 400

Leu Ala Gln Asn Ile Ala Gln Gln Gln Asn Ile Asp Lys Asn Gln Asp
405 410 415

Leu Leu Lys Gly Val Gly Thr Gln Ala Gly Leu Gly Ala Leu Tyr Gly
420 425 430

Phe Gly Ser Ala Gly Val Val Gln Ala Pro Ala Gly Ala Ala Arg Leu

435 440 445

Ala Gly Ala Ala Thr Ala Pro Val Leu Arg Thr Thr Met Ala Gly Val
450 455 460

Lys Ala Ala Gly Ser Val Ala Gly Lys Val Val Ser Pro Ile Lys Asn
465 470 475 480

Thr Leu Val Ala Arg Gly Glu Arg Val Met Lys Gln Asn Glu Glu Ala
485 490 495

Ser Pro Val Ala Asp Asp Tyr Val Ala Gln Ala Ala Gln Glu Ala Met
500 505 510

Ala Gln Ala Pro Glu Ala Glu Val Thr Ile Arg Asp Ala Val Glu Ala
515 520 525

Thr Asp Ala Thr Pro Glu Gln Lys Val Ala Ala His Gln Tyr Val Ser
530 535 540

Asp Leu Met Asn Ala Thr Arg Phe Asn Pro Glu Asn Tyr Gln Glu Ala
545 550 555 560

Pro Glu His Ile Arg Asn Ala Val Ala Gly Ser Thr Asp Gln Val Gln
565 570 575

Val Ile Gln Lys Leu Ala Asp Leu Val Asn Thr Leu Asp Glu Ser Asn
580 585 590

Pro Gln Ala Leu Met Glu Ala Ala Ser Tyr Met Tyr Asp Ala Val Ser
595 600 605

Glu Phe Glu Gln Phe Ile Asn Arg Asp Pro Ala Ala Leu Asp Ser Ile
610 615 620

Pro Lys Asp Ser Pro Ala Ile Glu Leu Leu Asn Arg Tyr Thr Asn Leu
625 630 635 640

Thr Ala Asn Ile Gln Asn Thr Pro Lys Val Ile Gly Ala Leu Asn Val
645 650 655

Ile Asn Arg Met Ile Asn Glu Ser Ala Gln Asn Gly Ser Leu Asn Val
660 665 670

Thr Glu Glu Ser Ser Pro Gln Glu Met Gln Asn Val Ala Leu Ala Ala
675 680 685

Glu Val Ala Pro Glu Lys Leu Asn Pro Glu Ser Val Asn Val Val Leu
690 695 700

Lys His Ala Ala Asp Gly Arg Ile Lys Leu Asn Asn Arg Gln Ile Ala
705 710 715 720

Ala Leu Gln Asn Ala Ala Ala Ile Leu Lys Gly Ala Arg Glu Tyr Asp
725 730 735

Ala Glu Ala Ala Arg Leu Gly Leu Arg Pro Gln Asp Ile Val Ser Lys
740 745 750

Gln Ile Lys Thr Asp Glu Ser Arg Thr Gln Glu Gly Gln Tyr Ser Ala

755 760 765

Leu Gln His Ala Asn Arg Ile Arg Ser Ala Tyr Asn Ser Gly Asn Phe
770 775 780

Glu Leu Ala Ser Ala Tyr Leu Asn Asp Phe Met Gln Phe Ala Gln His
785 790 795 800

Met Gln Asn Lys Val Gly Ala Leu Asn Glu His Leu Val Thr Gly Asn
805 810 815

Ala Asp Lys Asn Lys Ser Val His Tyr Gln Ala Leu Thr Ala Asp Arg
820 825 830

Glu Trp Val Arg Ser Arg Thr Gly Leu Gly Val Asn Pro Tyr Asp Thr
835 840 845

Lys Ser Val Lys Phe Ala Gln Gln Val Ala Leu Glu Ala Lys Thr Val
850 855 860

Ala Asp Ile Ala Asn Ala Leu Ala Ser Ala Tyr Pro Glu Leu Lys Val
865 870 875 880

Ser His Ile Lys Val Thr Pro Leu Asp Ser Arg Leu Asn Ala Pro Ala
885 890 895

Ala Glu Val Val Lys Ala Phe Arg Gln Gly Asn Arg Asp Val Ala Ser
900 905 910

Ser Gln Pro Lys Ala Asp Ser Val Asn Gln Val Lys Glu Thr Pro Val
915 920 925

Thr Lys Gln Glu Pro Val Thr Ser Thr Val Gln Thr Lys Thr Pro Val
930 935 940

Ser Glu Ser Val Lys Thr Glu Pro Thr Thr Lys Glu Ser Ser Pro Gln
945 950 955 960

Ala Ile Lys Glu Pro Val Asn Gln Ser Glu Lys Gln Asp Val Asn Leu
965 970 975

Thr Asn Glu Asp Asn Ile Lys Gln Pro Thr Glu Ser Val Lys Glu Thr
980 985 990

Glu Thr Ser Thr Lys Glu Ser Thr Val Thr Glu Glu Leu Lys Glu Gly
995 1000 1005

Ile Asp Ala Val Tyr Pro Ser Leu Val Gly Thr Ala Asp Ser Lys Ala
1010 1015 1020

Glu Gly Ile Lys Asn Tyr Phe Lys Leu Ser Phe Thr Leu Pro Glu Glu
1025 1030 1035 1040

Gln Lys Ser Arg Thr Val Gly Ser Glu Ala Pro Leu Lys Asp Val Ala
1045 1050 1055

Gln Ala Leu Ser Ser Arg Ala Arg Tyr Glu Leu Phe Thr Glu Lys Glu
1060 1065 1070

Thr Ala Asn Pro Ala Phe Asn Gly Glu Val Ile Lys Arg Tyr Lys Glu

1075 1080 1085

Leu Met Glu His Gly Glu Gly Ile Ala Asp Ile Leu Arg Ser Arg Leu
1090 1095 1100

Ala Lys Phe Leu Asn Thr Lys Asp Val Gly Lys Arg Phe Ala Gln Gly
1105 1110 1115 1120

Thr Glu Ala Asn Arg Trp Val Gly Gly Lys Leu Leu Asn Ile Val Glu
1125 1130 1135

Gln Asp Gly Asp Thr Phe Lys Tyr Asn Glu Gln Leu Leu Gln Thr Ala
1140 1145 1150

Val Leu Ala Gly Leu Gln Trp Arg Leu Thr Ala Thr Ser Asn Thr Ala
1155 1160 1165

Ile Lys Asp Ala Lys Asp Val Ala Ala Ile Thr Gly Ile Asp Gln Ala
1170 1175 1180

Leu Leu Pro Glu Gly Leu Val Glu Gln Phe Asp Thr Gly Met Thr Leu
1185 1190 1195 1200

Thr Glu Ala Val Ser Ser Leu Ala Gln Lys Ile Glu Ser Tyr Trp Gly
1205 1210 1215

Leu Ser Arg Asn Pro Asn Ala Pro Leu Gly Tyr Thr Lys Gly Ile Pro
1220 1225 1230

Thr Ala Met Ala Ala Glu Ile Leu Ala Ala Phe Val Glu Ser Thr Asp
1235 1240 1245

Val Val Glu Asn Ile Val Asp Met Ser Glu Ile Asp Pro Asp Asn Lys
1250 1255 1260

Lys Thr Ile Gly Leu Tyr Thr Ile Thr Glu Leu Asp Ser Phe Asp Pro
1265 1270 1275 1280

Ile Asn Ser Phe Pro Thr Ala Ile Glu Glu Ala Val Leu Val Asn Pro
1285 1290 1295

Thr Glu Lys Met Phe Phe Gly Asp Asp Ile Pro Pro Val Ala Asn Thr
1300 1305 1310

Gln Leu Arg Asn Pro Ala Val Arg Asn Thr Pro Glu Gln Lys Ala Ala
1315 1320 1325

Leu Lys Ala Glu Gln Ala Thr Glu Phe Tyr Val His Thr Pro Met Val
1330 1335 1340

Gln Phe Tyr Glu Thr Leu Gly Lys Asp Arg Ile Leu Glu Leu Met Gly
1345 1350 1355 1360

Ala Gly Thr Leu Asn Lys Glu Leu Leu Asn Asp Asn His Ala Lys Ser
1365 1370 1375

Leu Glu Gly Lys Asn Arg Ser Val Glu Asp Ser Tyr Asn Gln Leu Phe
1380 1385 1390

Ser Val Ile Glu Gln Val Arg Ala Gln Ser Glu Asp Ile Ser Thr Val

1395 1400 1405

Pro Ile His Tyr Ala Tyr Asn Met Thr Arg Val Gly Arg Met Gln Met
1410 1415 1420

Leu Gly Lys Tyr Asn Pro Gln Ser Ala Lys Leu Val Arg Glu Ala Ile
1425 1430 1435 1440

Leu Pro Thr Lys Ala Thr Leu Asp Leu Ser Asn Gln Asn Asn Glu Asp
1445 1450 1455

Phe Ser Ala Phe Gln Leu Gly Leu Ala Gln Ala Leu Asp Ile Lys Val
1460 1465 1470

His Thr Met Thr Arg Glu Val Met Ser Asp Glu Leu Thr Lys Leu Leu
1475 1480 1485

Glu Gly Asn Leu Lys Pro Ala Ile Asp Met Met Val Glu Phe Asn Thr
1490 1495 1500

Thr Gly Ser Leu Pro Glu Asn Ala Val Asp Val Leu Asn Thr Ala Leu
1505 1510 1515 1520

Gly Asp Arg Lys Ser Phe Val Ala Leu Met Ala Leu Met Glu Tyr Ser
1525 1530 1535

Arg Tyr Leu Val Ala Glu Asp Lys Ser Ala Phe Val Thr Pro Leu Tyr
1540 1545 1550

Val Glu Ala Asp Gly Val Thr Asn Gly Pro Ile Asn Ala Met Met Leu
1555 1560 1565

Met Thr Gly Gly Leu Phe Thr Pro Asp Trp Ile Arg Asn Ile Ala Lys
1570 1575 1580

Gly Gly Leu Phe Ile Gly Ser Pro Asn Lys Thr Met Asn Glu His Arg
1585 1590 1595 1600

Ser Thr Ala Asp Asn Asn Asp Leu Tyr Gln Ala Ser Thr Asn Ala Leu
1605 1610 1615

Met Glu Ser Leu Gly Lys Leu Arg Ser Asn Tyr Ala Ser Asn Met Pro
1620 1625 1630

Ile Gln Ser Gln Ile Asp Ser Leu Leu Ser Leu Met Asp Leu Phe Leu
1635 1640 1645

Pro Asp Ile Asn Leu Gly Glu Asn Gly Ala Leu Glu Leu Lys Arg Gly
1650 1655 1660

Ile Ala Lys Asn Pro Leu Thr Ile Thr Ile Tyr Gly Ser Gly Ala Arg
1665 1670 1675 1680

Gly Ile Ala Gly Lys Leu Val Ser Ser Val Thr Asp Ala Ile Tyr Glu
1685 1690 1695

Arg Met Ser Asp Val Leu Lys Ala Arg Ala Lys Asp Pro Asn Ile Ser
1700 1705 1710

Ala Ala Met Ala Met Phe Gly Lys Gln Ala Ala Ser Glu Ala His Ala

1715 1720 1725

Glu Glu Leu Leu Ala Arg Phe Leu Lys Asp Met Glu Thr Leu Thr Ser
1730 1735 1740

Thr Val Pro Val Lys Arg Lys Gly Val Leu Glu Leu Gln Ser Thr Gly
1745 1750 1755 1760

Thr Gly Ala Lys Gly Lys Ile Asn Pro Lys Thr Tyr Thr Ile Lys Gly
1765 1770 1775

Glu Gln Leu Lys Ala Leu Gln Glu Asn Met Leu His Phe Phe Val Glu
1780 1785 1790

Pro Leu Arg Asn Gly Ile Thr Gln Thr Val Gly Glu Ser Leu Val Tyr
1795 1800 1805

Ser Thr Glu Gln Leu Gln Lys Ala Thr Gln Ile Gln Ser Val Val Leu
1810 1815 1820

Glu Asp Met Phe Lys Gln Arg Val Gln Glu Lys Leu Ala Glu Lys Ala
1825 1830 1835 1840

Lys Asp Pro Thr Trp Lys Lys Gly Asp Phe Leu Thr Gln Lys Glu Leu
1845 1850 1855

Asn Asp Ile Gln Ala Ser Leu Asn Asn Leu Ala Pro Met Ile Glu Thr
1860 1865 1870

Gly Ser Gln Thr Phe Tyr Ile Ala Gly Ser Glu Asn Ala Glu Val Ala
1875 1880 1885

Asn Gln Val Leu Ala Thr Asn Leu Asp Asp Arg Met Arg Val Pro Met
1890 1895 1900

Ser Ile Tyr Ala Pro Ala Gln Ala Gly Val Ala Gly Ile Pro Phe Met
1905 1910 1915 1920

Thr Ile Gly Thr Gly Asp Gly Met Met Met Gln Thr Leu Ser Thr Met
1925 1930 1935

Lys Gly Ala Pro Lys Asn Thr Leu Lys Ile Phe Asp Gly Met Asn Ile
1940 1945 1950

Gly Leu Asn Asp Ile Thr Asp Ala Ser Arg Lys Ala Asn Glu Ala Val
1955 1960 1965

Tyr Thr Ser Trp Gln Gly Asn Pro Ile Lys Asn Val Tyr Glu Ser Tyr
1970 1975 1980

Ala Lys Phe Met Lys Asn Val Asp Phe Ser Lys Leu Ser Pro Glu Ala
1985 1990 1995 2000

Leu Glu Ala Ile Gly Lys Ser Ala Leu Glu Tyr Asp Gln Arg Glu Asn
2005 2010 2015

Ala Thr Val Asp Asp Ile Ala Asn Ala Ala Ser Leu Ile Glu Arg Asn
2020 2025 2030

Leu Arg Asn Ile Ala Leu Gly Val Asp Ile Arg His Lys Val Leu Asp

2035 2040 2045

Lys Val Asn Leu Ser Ile Asp Gln Met Ala Ala Val Gly Ala Pro Tyr
2050 2055 2060

Gln Asn Asn Gly Lys Ile Asp Leu Ser Asn Met Thr Pro Glu Gln Gln
2065 2070 2075 2080

Ala Asp Glu Leu Asn Lys Leu Phe Arg Glu Glu Leu Glu Ala Arg Lys
2085 2090 2095

Gln Lys Val Ala Lys Ala Arg Ala Glu Val Lys Glu Glu Thr Val Ser
2100 2105 2110

Glu Lys Glu Pro Val Asn Pro Asp Phe Gly Met Val Gly Arg Glu His
2115 2120 2125

Lys Ala Ser Gly Val Arg Ile Leu Ser Ala Thr Ala Ile Arg Asn Leu
2130 2135 2140

Ala Lys Ile Ser Asn Leu Pro Ser Thr Gln Ala Ala Thr Leu Ala Glu
2145 2150 2155 2160

Ile Gln Lys Ser Leu Ala Ala Lys Asp Tyr Lys Ile Ile Tyr Gly Thr
2165 2170 2175

Pro Thr Gln Val Ala Glu Tyr Ala Arg Gln Lys Asn Val Thr Glu Leu
2180 2185 2190

Thr Ser Gln Glu Met Glu Glu Ala Gln Ala Gly Asn Ile Tyr Gly Trp
2195 2200 2205

Thr Asn Phe Asp Asp Lys Thr Ile Tyr Leu Val Ser Pro Ser Met Glu
2210 2215 2220

Thr Leu Ile His Glu Leu Val His Ala Ser Thr Phe Glu Glu Val Tyr
2225 2230 2235 2240

Ser Phe Tyr Gln Gly Asn Glu Val Ser Pro Thr Ser Lys Gln Ala Ile
2245 2250 2255

Glu Asn Leu Glu Gly Leu Met Glu Gln Phe Arg Ser Leu Asp Ile Ser
2260 2265 2270

Lys Asp Ser Pro Glu Met Arg Glu Ala Tyr Ala Asp Ala Ile Ala Thr
2275 2280 2285

Ile Glu Gly His Leu Ser Asn Gly Phe Val Asp Pro Ala Ile Ser Lys
2290 2295 2300

Ala Ala Ala Leu Asn Glu Phe Met Ala Trp Gly Leu Ala Asn Arg Ala
2305 2310 2315 2320

Leu Ala Ala Lys Gln Lys Arg Thr Ser Ser Leu Val Gln Met Val Lys
2325 2330 2335

Asp Val Tyr Gln Ala Ile Lys Lys Leu Ile Trp Gly Arg Lys Gln Ala
2340 2345 2350

Pro Ala Leu Gly Glu Asp Met Phe Ser Asn Leu Leu Phe Asn Ser Ala

2355 2360 2365

Ile Leu Met Arg Ser Gln Pro Thr Thr Gln Ala Val Ala Lys Asp Gly
2370 2375 2380

Thr Leu Phe His Ser Lys Ala Tyr Gly Asn Asn Glu Arg Leu Ser Gln
2385 2390 2395 2400

Leu Asn Gln Thr Phe Asp Lys Leu Val Thr Asp Tyr Leu Arg Thr Asp
2405 2410 2415

Pro Val Thr Glu Val Glu Arg Arg Gly Asn Val Ala Asn Ala Leu Met
2420 2425 2430

Ser Ala Thr Arg Leu Val Arg Asp Val Gln Ser His Gly Phe Asn Met
2435 2440 2445

Thr Ala Gln Glu Gln Ser Val Phe Gln Met Val Thr Ala Ala Leu Ala
2450 2455 2460

Thr Glu Ala Ala Ile Asp Pro His Ala Met Ala Arg Ala Gln Glu Leu
2465 2470 2475 2480

Tyr Thr His Val Met Lys His Leu Thr Val Glu His Phe Met Ala Asp
2485 2490 2495

Pro Asp Ser Thr Asn Pro Ala Asp Arg Tyr Tyr Ala Gln Gln Lys Tyr
2500 2505 2510

Asp Thr Ile Ser Gly Ala Asn Leu Val Glu Val Asp Ala Lys Gly Arg
2515 2520 2525

Thr Ser Leu Leu Pro Thr Phe Leu Gly Leu Ala Met Val Asn Glu Glu
2530 2535 2540

Leu Arg Ser Ile Ile Lys Glu Met Pro Val Pro Lys Ala Asp Lys Lys
2545 2550 2555 2560

Leu Gly Asn Asp Ile Asp Thr Leu Leu Thr Asn Ala Gly Thr Gln Val
2565 2570 2575

Met Glu Ser Leu Asn Arg Arg Met Ala Gly Asp Gln Lys Ala Thr Asn
2580 2585 2590

Val Gln Asp Ser Ile Asp Ala Leu Ser Glu Thr Ile Met Ala Ala Ala
2595 2600 2605

Leu Lys Arg Glu Ser Phe Tyr Asp Ala Val Ala Thr Pro Thr Gly Asn
2610 2615 2620

Phe Ile Asp Arg Ala Asn Gln Tyr Val Thr Asp Ser Ile Glu Arg Leu
2625 2630 2635 2640

Ser Glu Thr Val Ile Glu Lys Ala Asp Lys Val Ile Ala Asn Pro Ser
2645 2650 2655

Asn Ile Ala Ala Lys Gly Val Ala His Leu Ala Lys Leu Thr Ala Ala
2660 2665 2670

Ile Ala Ser Glu Lys Gln Gly Glu Ile Val Ala Gln Gly Val Met Thr

2675 2680 2685

Ala Met Asn Gln Gly Lys Val Trp Gln Pro Phe His Asp Leu Val Asn
2690 2695 2700

Asp Ile Val Gly Arg Thr Lys Thr Asn Ala Asn Val Tyr Asp Leu Ile
2705 2710 2715 2720

Lys Leu Val Lys Ser Gln Ile Ser Gln Asp Arg Gln Gln Phe Arg Glu
2725 2730 2735

His Leu Pro Thr Val Ile Ala Gly Lys Phe Ser Arg Lys Leu Thr Asp
2740 2745 2750

Thr Glu Trp Ser Ala Met His Thr Gly Leu Gly Lys Thr Asp Leu Ala
2755 2760 2765

Val Leu Arg Glu Thr Met Ser Met Ala Glu Ile Arg Asp Leu Leu Ser
2770 2775 2780

Ser Ser Lys Lys Val Lys Asp Glu Ile Ser Thr Leu Glu Lys Glu Ile
2785 2790 2795 2800

Gln Asn Gln Ala Gly Arg Asn Trp Asn Leu Val Gln Lys Lys Ser Lys
2805 2810 2815

Gln Leu Ala Gln Tyr Met Ile Met Gly Glu Val Gly Asn Asn Leu Leu
2820 2825 2830

Arg Asn Ala His Ala Ile Ser Arg Leu Leu Gly Glu Arg Ile Thr Asn
2835 2840 2845

Gly Pro Val Ala Asp Val Ala Ala Ile Asp Lys Leu Ile Thr Leu Tyr
2850 2855 2860

Ser Leu Glu Leu Met Asn Lys Ser Asp Arg Asp Leu Leu Ser Glu Leu
2865 2870 2875 2880

Ala Gln Ser Glu Val Glu Gly Met Glu Phe Ser Ile Ala Tyr Met Val
2885 2890 2895

Gly Gln Arg Thr Glu Glu Met Arg Lys Ala Lys Gly Asp Asn Arg Thr
2900 2905 2910

Leu Leu Asn His Phe Lys Gly Tyr Ile Pro Val Glu Asn Gln Gln Gly
2915 2920 2925

Val Asn Leu Ile Ile Ala Asp Asp Lys Glu Phe Ala Lys Leu Asn Ser
2930 2935 2940

Gln Ser Phe Thr Arg Ile Gly Thr Tyr Gln Gly Ser Thr Gly Phe Arg
2945 2950 2955 2960

Thr Gly Ser Lys Gly Tyr Tyr Phe Ser Pro Val Ala Ala Arg Ala Pro
2965 2970 2975

Tyr Ser Gln Gly Ile Leu Gln Asn Val Arg Asn Thr Ala Gly Gly Val
2980 2985 2990

Asp Ile Gly Thr Gly Phe Thr Leu Gly Thr Met Val Ala Gly Arg Ile

2995 3000 3005

Thr Asp Lys Pro Thr Val Glu Arg Ile Thr Lys Ala Leu Ala Lys Gly
3010 3015 3020

Glu Arg Gly Arg Glu Pro Leu Met Pro Ile Tyr Asn Ser Lys Gly Gln
3025 3030 3035 3040

Val Val Ala Tyr Glu Gln Ser Val Asp Pro Asn Met Leu Lys His Leu
3045 3050 3055

Asn Gln Asp Asn His Phe Ala Lys Met Val Gly Val Trp Arg Gly Arg
3060 3065 3070

Gln Val Glu Glu Ala Lys Ala Gln Arg Phe Asn Asp Ile Leu Ile Glu
3075 3080 3085

Gln Leu His Ala Met Tyr Glu Lys Asp Ile Lys Asp Ser Ser Ala Asn
3090 3095 3100

Lys Ser Gln Tyr Val Asn Leu Leu Gly Lys Ile Asp Asp Pro Val Leu
3105 3110 3115 3120

Ala Asp Ala Ile Asn Leu Met Asn Ile Glu Thr Arg His Lys Ala Glu
3125 3130 3135

Glu Leu Phe Gly Lys Asp Glu Leu Trp Val Arg Arg Asp Met Leu Asn
3140 3145 3150

Asp Ala Leu Gly Tyr Arg Ala Ala Ser Ile Gly Asp Val Trp Thr Gly
3155 3160 3165

Asn Ser Arg Trp Ser Pro Ser Thr Leu Asp Thr Val Lys Lys Met Phe
3170 3175 3180

Leu Gly Ala Phe Gly Asn Lys Ala Tyr His Val Val Met Asn Ala Glu
3185 3190 3195 3200

Asn Thr Ile Gln Asn Leu Val Lys Asp Ala Lys Thr Val Ile Val Val
3205 3210 3215

Lys Ser Val Val Val Pro Ala Val Asn Phe Leu Ala Asn Ile Tyr Gln
3220 3225 3230

Met Ile Gly Arg Gly Val Pro Val Lys Asp Ile Ala Val Asn Ile Pro
3235 3240 3245

Arg Lys Thr Ser Glu Ile Asn Gln Tyr Ile Lys Ser Arg Leu Arg Gln
3250 3255 3260

Ile Asp Ala Glu Ala Glu Leu Arg Ala Ala Glu Gly Asn Pro Asn Leu
3265 3270 3275 3280

Val Arg Lys Leu Lys Thr Glu Ile Gln Ser Ile Thr Asp Ser His Arg
3285 3290 3295

Arg Met Ser Ile Trp Pro Leu Ile Glu Ala Gly Glu Phe Ser Ser Ile
3300 3305 3310

Ala Asp Ala Gly Ile Ser Arg Asp Asp Leu Leu Val Ala Glu Gly Lys

3315 3320 3325

Ile His Glu Tyr Met Glu Lys Leu Ala Asn Lys Leu Pro Glu Lys Val
3330 3335 3340

Arg Asn Ala Gly Arg Tyr Ala Leu Ile Ala Lys Asp Thr Ala Leu Phe
3345 3350 3355 3360

Gln Gly Ile Gln Lys Thr Val Glu Tyr Ser Asp Phe Ile Ala Lys Ala
3365 3370 3375

Ile Ile Tyr Asp Asp Leu Val Lys Arg Lys Lys Lys Ser Ser Ser Glu
3380 3385 3390

Ala Leu Gly Gln Val Thr Glu Glu Phe Ile Asn Tyr Asp Arg Leu Pro
3395 3400 3405

Gly Arg Phe Arg Gly Tyr Met Glu Ser Met Gly Leu Met Trp Phe Tyr
3410 3415 3420

Asn Phe Lys Ile Arg Ser Ile Lys Val Ala Met Ser Met Ile Arg Asn
3425 3430 3435 3440

Asn Pro Val His Ser Leu Ile Ala Thr Val Val Pro Ala Pro Thr Met
3445 3450 3455

Phe Gly Asn Val Gly Leu Pro Ile Gln Asp Asn Met Leu Thr Met Leu
3460 3465 3470

Ala Glu Gly Arg Leu Asp Tyr Ser Leu Gly Phe Gly Gln Gly Leu Arg
3475 3480 3485

Ala Pro Thr Leu Asn Pro Trp Phe Asn Leu Thr His
3490 3495 3500

<210> 3
<211> 3318
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 3
gaaagtacag ttacagaaga attaaaagaa ggtattgatg ctgtttaccc ttcattggta 60
ggtactgctg attctaaagc agagggtatt aagaactatt tcaaattgtc ctttacctta 120
ccagaagaac agaaatcccg tactgttggt tcagaagcac ctctaaaaga tgtagcccaa 180
gctctgtctt ctcgtgctcg ttatgaactc tttactgaga aagaaactgc taaccctgct 240
tttaatgggg aagttattaa gcgatacaaa gaactcatgg aacatgggga aggtattgct 300
gatattcttc gctcccgtct ggctaagttc cttaacacta aggatgttgg taaacgtttt 360
gctcaaggta cagaagccaa ccgttgggta ggtggtaagt tacttaacat tgttgagcag 420
gatggggata cctttaagta caacgaacaa ttgctacaga ctgctgtatt agcaggtctt 480
caatggagac ttactgctac cagcaatact gctatcaaag atgcaaaaga tgttgctgct 540
attactggta ttgaccaagc tctgctgcca gaaggtttag tagagcaatt tgatactggt 600
atgacactca ctgaagcagt tagttccctg gctcagaaaa ttgagtctta ctggggatta 660
tctcgtaatc caaatgctcc attgggctat accaaaggca tccctacagc aatggctgct 720
gaaattctgg ctgcatttgt agagtctact gatgttgtag agaacatcgt ggatatgtca 780
gaaattgacc cagataacaa gaagactatt ggtctgtaca ccattactga actggattcc 840

ttcgacccaa ttaatagctt ccctactgct attgaagaag ctgttttagt gaatcctaca 900
gagaagatgt tctttggtga tgacattcct cctgtagcta atactcagct tcgtaaccct 960
gctgttcgta atactccaga acagaaggct gcattgaaag cagagcaggc tacagagttc 1020
tatgtacaca ccccaatggt tcaattctat gagacgttag gtaaagaccg tattctcgaa 1080
ctgatgggtg ctggtactct gaataaagag ttacttaatg ataaccatgc taaatctctg 1140
gaaggtaaga accgttcagt agaggactct tacaaccaac tgttctccgt cattgagcag 1200
gtaagagcac agagcgaaga catctctact gtacctattc actatgcata caatatgacc 1260
cgtgttggtc gtatgcagat gttaggtaaa tacaatcctc aatcagccaa actggttcgt 1320
gaggccatct tacctactaa agctactttg gatttatcga accagaacaa tgaagacttc 1380
tctgcattcc agttaggtct ggctcaggca ttggacatta aagtccatac tatgactcgt 1440
gaggttatgt ctgacgagtt gactaaatta ctggaaggta atctgaaacc agccattgat 1500
atgatggttg agtttaatac cactggttcc ttaccagaaa acgcagttga tgttctgaat 1560
acagcattag gagataggaa gtcattcgta gcattgatgg ctcttatgga gtattcccgt 1620
tacttagtag cagaggataa atctgcattt gtaactccac tgtatgtaga agcagatggt 1680
gttactaatg gtccaatcaa tgccatgatg ctaatgacag gcggtctgtt tactcctgac 1740
tggattcgta atattgccaa aggggggcttg ttcattggtt ctccaaataa gaccatgaat 1800
gagcatcgct ctactgctga caataatgat ttatatcaag catccactaa tgctttgatg 1860
gaatcgttgg gtaagttacg tagtaactat gcctctaata tgcctattca gtctcagata 1920
gacagtcttc tttctctgat ggatttgttt ttaccggata ttaatcttgg tgagaatggt 1980
gctttagaac ttaaacgtgg tattgctaag aacccactga ctattaccat ctatggttct 2040
ggtgctcgtg gtattgcagg taagctggtt agttctgtta ctgatgccat ctatgagcgt 2100
atgtctgatg tactgaaagc tcgtgctaaa gacccaaata tctctgctgc tatggcaatg 2160
tttggtaagc aagctgcttc agaagcacat gctgaagaac ttcttgcccg tttcctgaaa 2220
gatatggaaa cactgacttc tactgttcct gttaaacgta aaggtgtact ggaactacaa 2280
tccacaggta caggagccaa aggaaaaatc aatcctaaga cctataccat taagggcgag 2340
caactgaagg cacttcagga aaatatgctg cacttctttg tagaaccact acgtaatggt 2400
attactcaga ctgtaggtga aagtctggtg tactctactg aacaattaca gaaagctact 2460
cagattcaat ctgtagtgct ggaagatatg ttcaaacagc gagtacaaga gaagctggca 2520
gagaaggcta aagacccaac atggaagaaa ggtgatttcc ttactcagaa agaactgaat 2580
gatattcagg cttctctgaa taacttagcc cctatgattg agactggttc tcagactttc 2640
tacattgctg gttcagaaaa tgcagaagta gcaaatcagg tattagctac taaccttgat 2700
gaccgtatgc gtgtaccaat gagtatctat gctccagcac aggccggtgt agcaggtatt 2760
ccatttatga ctattggtac tggtgatggc atgatgatgc aaactctttc cactatgaaa 2820
ggtgcaccaa agaataccct caaaatcttt gatggtatga acattggttt gaatgacatc 2880
actgatgcca gtcgtaaagc taatgaagct gtttacactt cttggcaggg taaccctatt 2940
aagaatgttt atgaatcata tgctaagttc atgaagaatg tagatttcag caagctgtcc 3000
cctgaagcat tggaagcaat tggtaaatct gctctggaat atgaccaacg tgagaatgct 3060
actgtagatg atattgctaa cgctgcatct ctgattgaac gtaacttacg taatattgca 3120
ctgggtgtag atattcgtca taaggtgctg gataaggtaa atctgtccat tgaccagatg 3180
gctgctgtag gtgctcctta tcagaacaac ggtaagattg acctcagcaa tatgacccct 3240
gaacaacagg ctgatgaact gaataaactt ttccgtgaag agttagaagc ccgtaaacaa 3300
aaagtcgcta aggctagg 3318

<210> 4
<211> 1107
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 4
Met Glu Ser Thr Val Thr Glu Glu Leu Lys Glu Gly Ile Asp Ala Val
1 5 10 15

Tyr Pro Ser Leu Val Gly Thr Ala Asp Ser Lys Ala Glu Gly Ile Lys
20 25 30

```
Asn Tyr Phe Lys Leu Ser Phe Thr Leu Pro Glu Glu Gln Lys Ser Arg
        35                  40                  45

Thr Val Gly Ser Glu Ala Pro Leu Lys Asp Val Ala Gln Ala Leu Ser
    50                  55                  60

Ser Arg Ala Arg Tyr Glu Leu Phe Thr Glu Lys Glu Thr Ala Asn Pro
65                  70                  75                  80

Ala Phe Asn Gly Glu Val Ile Lys Arg Tyr Lys Glu Leu Met Glu His
                85                  90                  95

Gly Glu Gly Ile Ala Asp Ile Leu Arg Ser Arg Leu Ala Lys Phe Leu
            100                 105                 110

Asn Thr Lys Asp Val Gly Lys Arg Phe Ala Gln Gly Thr Glu Ala Asn
        115                 120                 125

Arg Trp Val Gly Gly Lys Leu Leu Asn Ile Val Glu Gln Asp Gly Asp
    130                 135                 140

Thr Phe Lys Tyr Asn Glu Gln Leu Leu Gln Thr Ala Val Leu Ala Gly
145                 150                 155                 160

Leu Gln Trp Arg Leu Thr Ala Thr Ser Asn Thr Ala Ile Lys Asp Ala
                165                 170                 175

Lys Asp Val Ala Ala Ile Thr Gly Ile Asp Gln Ala Leu Leu Pro Glu
            180                 185                 190

Gly Leu Val Glu Gln Phe Asp Thr Gly Met Thr Leu Thr Glu Ala Val
        195                 200                 205

Ser Ser Leu Ala Gln Lys Ile Glu Ser Tyr Trp Gly Leu Ser Arg Asn
    210                 215                 220

Pro Asn Ala Pro Leu Gly Tyr Thr Lys Gly Ile Pro Thr Ala Met Ala
225                 230                 235                 240

Ala Glu Ile Leu Ala Ala Phe Val Glu Ser Thr Asp Val Val Glu Asn
                245                 250                 255

Ile Val Asp Met Ser Glu Ile Asp Pro Asp Asn Lys Lys Thr Ile Gly
            260                 265                 270

Leu Tyr Thr Ile Thr Glu Leu Asp Ser Phe Asp Pro Ile Asn Ser Phe
        275                 280                 285

Pro Thr Ala Ile Glu Glu Ala Val Leu Val Asn Pro Thr Glu Lys Met
    290                 295                 300

Phe Phe Gly Asp Asp Ile Pro Pro Val Ala Asn Thr Gln Leu Arg Asn
305                 310                 315                 320

Pro Ala Val Arg Asn Thr Pro Glu Gln Lys Ala Ala Leu Lys Ala Glu
                325                 330                 335

Gln Ala Thr Glu Phe Tyr Val His Thr Pro Met Val Gln Phe Tyr Glu
            340                 345                 350
```

Thr Leu Gly Lys Asp Arg Ile Leu Glu Leu Met Gly Ala Gly Thr Leu
355 360 365

Asn Lys Glu Leu Leu Asn Asp Asn His Ala Lys Ser Leu Glu Gly Lys
370 375 380

Asn Arg Ser Val Glu Asp Ser Tyr Asn Gln Leu Phe Ser Val Ile Glu
385 390 395 400

Gln Val Arg Ala Gln Ser Glu Asp Ile Ser Thr Val Pro Ile His Tyr
405 410 415

Ala Tyr Asn Met Thr Arg Val Gly Arg Met Gln Met Leu Gly Lys Tyr
420 425 430

Asn Pro Gln Ser Ala Lys Leu Val Arg Glu Ala Ile Leu Pro Thr Lys
435 440 445

Ala Thr Leu Asp Leu Ser Asn Gln Asn Asn Glu Asp Phe Ser Ala Phe
450 455 460

Gln Leu Gly Leu Ala Gln Ala Leu Asp Ile Lys Val His Thr Met Thr
465 470 475 480

Arg Glu Val Met Ser Asp Glu Leu Thr Lys Leu Leu Glu Gly Asn Leu
485 490 495

Lys Pro Ala Ile Asp Met Met Val Glu Phe Asn Thr Thr Gly Ser Leu
500 505 510

Pro Glu Asn Ala Val Asp Val Leu Asn Thr Ala Leu Gly Asp Arg Lys
515 520 525

Ser Phe Val Ala Leu Met Ala Leu Met Glu Tyr Ser Arg Tyr Leu Val
530 535 540

Ala Glu Asp Lys Ser Ala Phe Val Thr Pro Leu Tyr Val Glu Ala Asp
545 550 555 560

Gly Val Thr Asn Gly Pro Ile Asn Ala Met Met Leu Met Thr Gly Gly
565 570 575

Leu Phe Thr Pro Asp Trp Ile Arg Asn Ile Ala Lys Gly Gly Leu Phe
580 585 590

Ile Gly Ser Pro Asn Lys Thr Met Asn Glu His Arg Ser Thr Ala Asp
595 600 605

Asn Asn Asp Leu Tyr Gln Ala Ser Thr Asn Ala Leu Met Glu Ser Leu
610 615 620

Gly Lys Leu Arg Ser Asn Tyr Ala Ser Asn Met Pro Ile Gln Ser Gln
625 630 635 640

Ile Asp Ser Leu Leu Ser Leu Met Asp Leu Phe Leu Pro Asp Ile Asn
645 650 655

Leu Gly Glu Asn Gly Ala Leu Glu Leu Lys Arg Gly Ile Ala Lys Asn
660 665 670

Pro Leu Thr Ile Thr Ile Tyr Gly Ser Gly Ala Arg Gly Ile Ala Gly
675 680 685

Lys Leu Val Ser Ser Val Thr Asp Ala Ile Tyr Glu Arg Met Ser Asp
690 695 700

Val Leu Lys Ala Arg Ala Lys Asp Pro Asn Ile Ser Ala Ala Met Ala
705 710 715 720

Met Phe Gly Lys Gln Ala Ala Ser Glu Ala His Ala Glu Glu Leu Leu
725 730 735

Ala Arg Phe Leu Lys Asp Met Glu Thr Leu Thr Ser Thr Val Pro Val
740 745 750

Lys Arg Lys Gly Val Leu Glu Leu Gln Ser Thr Gly Thr Gly Ala Lys
755 760 765

Gly Lys Ile Asn Pro Lys Thr Tyr Thr Ile Lys Gly Glu Gln Leu Lys
770 775 780

Ala Leu Gln Glu Asn Met Leu His Phe Phe Val Glu Pro Leu Arg Asn
785 790 795 800

Gly Ile Thr Gln Thr Val Gly Glu Ser Leu Val Tyr Ser Thr Glu Gln
805 810 815

Leu Gln Lys Ala Thr Gln Ile Gln Ser Val Val Leu Glu Asp Met Phe
820 825 830

Lys Gln Arg Val Gln Glu Lys Leu Ala Glu Lys Ala Lys Asp Pro Thr
835 840 845

Trp Lys Lys Gly Asp Phe Leu Thr Gln Lys Glu Leu Asn Asp Ile Gln
850 855 860

Ala Ser Leu Asn Asn Leu Ala Pro Met Ile Glu Thr Gly Ser Gln Thr
865 870 875 880

Phe Tyr Ile Ala Gly Ser Glu Asn Ala Glu Val Ala Asn Gln Val Leu
885 890 895

Ala Thr Asn Leu Asp Asp Arg Met Arg Val Pro Met Ser Ile Tyr Ala
900 905 910

Pro Ala Gln Ala Gly Val Ala Gly Ile Pro Phe Met Thr Ile Gly Thr
915 920 925

Gly Asp Gly Met Met Met Gln Thr Leu Ser Thr Met Lys Gly Ala Pro
930 935 940

Lys Asn Thr Leu Lys Ile Phe Asp Gly Met Asn Ile Gly Leu Asn Asp
945 950 955 960

Ile Thr Asp Ala Ser Arg Lys Ala Asn Glu Ala Val Tyr Thr Ser Trp
965 970 975

Gln Gly Asn Pro Ile Lys Asn Val Tyr Glu Ser Tyr Ala Lys Phe Met
980 985 990

Lys Asn Val Asp Phe Ser Lys Leu Ser Pro Glu Ala Leu Glu Ala Ile
995 1000 1005

Gly Lys Ser Ala Leu Glu Tyr Asp Gln Arg Glu Asn Ala Thr Val Asp
1010 1015 1020

Asp Ile Ala Asn Ala Ala Ser Leu Ile Glu Arg Asn Leu Arg Asn Ile
1025 1030 1035 1040

Ala Leu Gly Val Asp Ile Arg His Lys Val Leu Asp Lys Val Asn Leu
1045 1050 1055

Ser Ile Asp Gln Met Ala Ala Val Gly Ala Pro Tyr Gln Asn Asn Gly
1060 1065 1070

Lys Ile Asp Leu Ser Asn Met Thr Pro Glu Gln Gln Ala Asp Glu Leu
1075 1080 1085

Asn Lys Leu Phe Arg Glu Glu Leu Glu Ala Arg Lys Gln Lys Val Ala
1090 1095 1100

Lys Ala Arg
1105

<210> 5
<211> 3432
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 5
atggggggtt ctcatcatca tcatcatcat ggtatggcta gcatgactgg tggacagcaa 60
atgggtcggg atctgtacga cgatgacgat aaggatccga gctcgagatc tgaaagtaca 120
gttacagaag aattaaaaga aggtattgat gctgtttacc cttcattggt aggtactgct 180
gattctaaag cagagggtat taagaactat ttcaaattgt cctttacctt accagaagaa 240
cagaaatccc gtactgttgg ttcagaagca cctctaaaag atgtagccca agctctgtct 300
tctcgtgctc gttatgaact ctttactgag aaagaaactg ctaaccctgc ttttaatggg 360
gaagttatta agcgatacaa agaactcatg gaacatgggg aaggtattgc tgatattctt 420
cgctcccgtc tggctaagtt ccttaacact aaggatgttg gtaaacgttt tgctcaaggt 480
acagaagcca accgttgggt aggtggtaag ttacttaaca ttgttgagca ggatggggat 540
acctttaagt acaacgaaca attgctacag actgctgtat tagcaggtct tcaatggaga 600
cttactgcta ccagcaatac tgctatcaaa gatgcaaaag atgttgctgc tattactggt 660
attgaccaag ctctgctgcc agaaggttta gtagagcaat ttgatactgg tatgacactc 720
actgaagcag ttagttccct ggctcagaaa attgagtctt actggggatt atctcgtaat 780
ccaaatgctc cattgggcta taccaaaggc atccctacag caatggctgc tgaaattctg 840
gctgcatttg tagagtctac tgatgttgta gagaacatcg tggatatgtc agaaattgac 900
ccagataaca agaagactat tggtctgtac accattactg aactggattc cttcgaccca 960
attaatagct tccctactgc tattgaagaa gctgttttag tgaatcctac agagaagatg 1020
ttctttggtg atgacattcc tcctgtagct aatactcagc ttcgtaaccc tgctgttcgt 1080
aatactccag aacagaaggc tgcattgaaa gcagagcagg ctacagagtt ctatgtacac 1140
accccaatgg ttcaattcta tgagacgtta ggtaaagacc gtattctcga actgatgggt 1200
gctggtactc tgaataaaga gttacttaat gataaccatg ctaaatctct ggaaggtaag 1260
aaccgttcag tagaggactc ttacaaccaa ctgttctccg tcattgagca ggtaagagca 1320
cagagcgaag acatctctac tgtacctatt cactatgcat acaatatgac ccgtgttggt 1380
cgtatgcaga tgttaggtaa atacaatcct caatcagcca aactggttcg tgaggccatc 1440
ttacctacta aagctacttt ggatttatcg aaccagaaca atgaagactt ctctgcattc 1500

cagttaggtc tggctcaggc attggacatt aaagtccata ctatgactcg tgaggttatg 1560
tctgacgagt tgactaaatt actggaaggt aatctgaaac cagccattga tatgatggtt 1620
gagtttaata ccactggttc cttaccagaa aacgcagttg atgttctgaa tacagcatta 1680
ggagatagga agtcattcgt agcattgatg gctcttatgg agtattcccg ttacttagta 1740
gcagaggata aatctgcatt tgtaactcca ctgtatgtag aagcagatgg tgttactaat 1800
ggtccaatca atgccatgat gctaatgaca ggcggtctgt ttactcctga ctggattcgt 1860
aatattgcca aagggggctt gttcattggt tctccaaata agaccatgaa tgagcatcgc 1920
tctactgctg acaataatga tttatatcaa gcatccacta atgctttgat ggaatcgttg 1980
ggtaagttac gtagtaacta tgcctctaat atgcctattc agtctcagat agacagtctt 2040
ctttctctga tggatttgtt tttaccggat attaatcttg gtgagaatgg tgctttagaa 2100
cttaaacgtg gtattgctaa gaacccactg actattacca tctatggttc tggtgctcgt 2160
ggtattgcag gtaagctggt tagttctgtt actgatgcca tctatgagcg tatgtctgat 2220
gtactgaaag ctcgtgctaa agacccaaat atctctgctg ctatggcaat gtttggtaag 2280
caagctgctt cagaagcaca tgctgaagaa cttcttgccc gtttcctgaa agatatggaa 2340
acactgactt ctactgttcc tgttaaacgt aaaggtgtac tggaactaca atccacaggt 2400
acaggagcca aaggaaaaat caatcctaag acctatacca ttaagggcga gcaactgaag 2460
gcacttcagg aaaatatgct gcacttcttt gtagaaccac tacgtaatgg tattactcag 2520
actgtaggtg aaagtctggt gtactctact gaacaattac agaaagctac tcagattcaa 2580
tctgtagtgc tggaagatat gttcaaacag cgagtacaag agaagctggc agagaaggct 2640
aaagacccaa catggaagaa aggtgatttc cttactcaga aagaactgaa tgatattcag 2700
gcttctctga ataacttagc ccctatgatt gagactggtt ctcagacttt ctacattgct 2760
ggttcagaaa atgcagaagt agcaaatcag gtattagcta ctaaccttga tgaccgtatg 2820
cgtgtaccaa tgagtatcta tgctccagca caggccggtg tagcaggtat tccatttatg 2880
actattggta ctggtgatgg catgatgatg caaactcttt ccactatgaa aggtgcacca 2940
aagaataccc tcaaaatctt tgatggtatg aacattggtt tgaatgacat cactgatgcc 3000
agtcgtaaag ctaatgaagc tgtttacact tcttggcagg gtaaccctat taagaatgtt 3060
tatgaatcat atgctaagtt catgaagaat gtagatttca gcaagctgtc ccctgaagca 3120
ttggaagcaa ttggtaaatc tgctctggaa tatgaccaac gtgagaatgc tactgtagat 3180
gatattgcta acgctgcatc tctgattgaa cgtaacttac gtaatattgc actgggtgta 3240
gatattcgtc ataaggtgct ggataaggta aatctgtcca ttgaccagat ggctgctgta 3300
ggtgctcctt atcagaacaa cggtaagatt gacctcagca atatgacccc tgaacaacag 3360
gctgatgaac tgaataaact tttccgtgaa gagttagaag cccgtaaaca aaaagtcgct 3420
aaggctaggt aa 3432

<210> 6
<211> 1143
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 6
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr
1 5 10 15

Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp
20 25 30

Pro Ser Ser Arg Ser Glu Ser Thr Val Thr Glu Glu Leu Lys Glu Gly
35 40 45

Ile Asp Ala Val Tyr Pro Ser Leu Val Gly Thr Ala Asp Ser Lys Ala
50 55 60

Glu Gly Ile Lys Asn Tyr Phe Lys Leu Ser Phe Thr Leu Pro Glu Glu
65 70 75 80

Gln Lys Ser Arg Thr Val Gly Ser Glu Ala Pro Leu Lys Asp Val Ala
85 90 95

Gln Ala Leu Ser Ser Arg Ala Arg Tyr Glu Leu Phe Thr Glu Lys Glu
100 105 110

Thr Ala Asn Pro Ala Phe Asn Gly Glu Val Ile Lys Arg Tyr Lys Glu
115 120 125

Leu Met Glu His Gly Glu Gly Ile Ala Asp Ile Leu Arg Ser Arg Leu
130 135 140

Ala Lys Phe Leu Asn Thr Lys Asp Val Gly Lys Arg Phe Ala Gln Gly
145 150 155 160

Thr Glu Ala Asn Arg Trp Val Gly Gly Lys Leu Leu Asn Ile Val Glu
165 170 175

Gln Asp Gly Asp Thr Phe Lys Tyr Asn Glu Gln Leu Leu Gln Thr Ala
180 185 190

Val Leu Ala Gly Leu Gln Trp Arg Leu Thr Ala Thr Ser Asn Thr Ala
195 200 205

Ile Lys Asp Ala Lys Asp Val Ala Ala Ile Thr Gly Ile Asp Gln Ala
210 215 220

Leu Leu Pro Glu Gly Leu Val Glu Gln Phe Asp Thr Gly Met Thr Leu
225 230 235 240

Thr Glu Ala Val Ser Ser Leu Ala Gln Lys Ile Glu Ser Tyr Trp Gly
245 250 255

Leu Ser Arg Asn Pro Asn Ala Pro Leu Gly Tyr Thr Lys Gly Ile Pro
260 265 270

Thr Ala Met Ala Ala Glu Ile Leu Ala Ala Phe Val Glu Ser Thr Asp
275 280 285

Val Val Glu Asn Ile Val Asp Met Ser Glu Ile Asp Pro Asp Asn Lys
290 295 300

Lys Thr Ile Gly Leu Tyr Thr Ile Thr Glu Leu Asp Ser Phe Asp Pro
305 310 315 320

Ile Asn Ser Phe Pro Thr Ala Ile Glu Glu Ala Val Leu Val Asn Pro
325 330 335

Thr Glu Lys Met Phe Phe Gly Asp Asp Ile Pro Pro Val Ala Asn Thr
340 345 350

Gln Leu Arg Asn Pro Ala Val Arg Asn Thr Pro Glu Gln Lys Ala Ala
355 360 365

Leu Lys Ala Glu Gln Ala Thr Glu Phe Tyr Val His Thr Pro Met Val
370 375 380

Gln Phe Tyr Glu Thr Leu Gly Lys Asp Arg Ile Leu Glu Leu Met Gly
385 390 395 400

Ala Gly Thr Leu Asn Lys Glu Leu Leu Asn Asp Asn His Ala Lys Ser
405 410 415

Leu Glu Gly Lys Asn Arg Ser Val Glu Asp Ser Tyr Asn Gln Leu Phe
420 425 430

Ser Val Ile Glu Gln Val Arg Ala Gln Ser Glu Asp Ile Ser Thr Val
435 440 445

Pro Ile His Tyr Ala Tyr Asn Met Thr Arg Val Gly Arg Met Gln Met
450 455 460

Leu Gly Lys Tyr Asn Pro Gln Ser Ala Lys Leu Val Arg Glu Ala Ile
465 470 475 480

Leu Pro Thr Lys Ala Thr Leu Asp Leu Ser Asn Gln Asn Asn Glu Asp
485 490 495

Phe Ser Ala Phe Gln Leu Gly Leu Ala Gln Ala Leu Asp Ile Lys Val
500 505 510

His Thr Met Thr Arg Glu Val Met Ser Asp Glu Leu Thr Lys Leu Leu
515 520 525

Glu Gly Asn Leu Lys Pro Ala Ile Asp Met Met Val Glu Phe Asn Thr
530 535 540

Thr Gly Ser Leu Pro Glu Asn Ala Val Asp Val Leu Asn Thr Ala Leu
545 550 555 560

Gly Asp Arg Lys Ser Phe Val Ala Leu Met Ala Leu Met Glu Tyr Ser
565 570 575

Arg Tyr Leu Val Ala Glu Asp Lys Ser Ala Phe Val Thr Pro Leu Tyr
580 585 590

Val Glu Ala Asp Gly Val Thr Asn Gly Pro Ile Asn Ala Met Met Leu
595 600 605

Met Thr Gly Gly Leu Phe Thr Pro Asp Trp Ile Arg Asn Ile Ala Lys
610 615 620

Gly Gly Leu Phe Ile Gly Ser Pro Asn Lys Thr Met Asn Glu His Arg
625 630 635 640

Ser Thr Ala Asp Asn Asn Asp Leu Tyr Gln Ala Ser Thr Asn Ala Leu
645 650 655

Met Glu Ser Leu Gly Lys Leu Arg Ser Asn Tyr Ala Ser Asn Met Pro
660 665 670

Ile Gln Ser Gln Ile Asp Ser Leu Leu Ser Leu Met Asp Leu Phe Leu
675 680 685

Pro Asp Ile Asn Leu Gly Glu Asn Gly Ala Leu Glu Leu Lys Arg Gly
690 695 700

Ile Ala Lys Asn Pro Leu Thr Ile Thr Ile Tyr Gly Ser Gly Ala Arg
705 710 715 720

```
Gly Ile Ala Gly Lys Leu Val Ser Ser Val Thr Asp Ala Ile Tyr Glu
                725                 730                 735

Arg Met Ser Asp Val Leu Lys Ala Arg Ala Lys Asp Pro Asn Ile Ser
            740                 745                 750

Ala Ala Met Ala Met Phe Gly Lys Gln Ala Ala Ser Glu Ala His Ala
        755                 760                 765

Glu Glu Leu Leu Ala Arg Phe Leu Lys Asp Met Glu Thr Leu Thr Ser
    770                 775                 780

Thr Val Pro Val Lys Arg Lys Gly Val Leu Glu Leu Gln Ser Thr Gly
785                 790                 795                 800

Thr Gly Ala Lys Gly Lys Ile Asn Pro Lys Thr Tyr Thr Ile Lys Gly
                805                 810                 815

Glu Gln Leu Lys Ala Leu Gln Glu Asn Met Leu His Phe Phe Val Glu
            820                 825                 830

Pro Leu Arg Asn Gly Ile Thr Gln Thr Val Gly Glu Ser Leu Val Tyr
        835                 840                 845

Ser Thr Glu Gln Leu Gln Lys Ala Thr Gln Ile Gln Ser Val Val Leu
    850                 855                 860

Glu Asp Met Phe Lys Gln Arg Val Gln Glu Lys Leu Ala Glu Lys Ala
865                 870                 875                 880

Lys Asp Pro Thr Trp Lys Lys Gly Asp Phe Leu Thr Gln Lys Glu Leu
                885                 890                 895

Asn Asp Ile Gln Ala Ser Leu Asn Asn Leu Ala Pro Met Ile Glu Thr
            900                 905                 910

Gly Ser Gln Thr Phe Tyr Ile Ala Gly Ser Glu Asn Ala Glu Val Ala
        915                 920                 925

Asn Gln Val Leu Ala Thr Asn Leu Asp Asp Arg Met Arg Val Pro Met
    930                 935                 940

Ser Ile Tyr Ala Pro Ala Gln Ala Gly Val Ala Gly Ile Pro Phe Met
945                 950                 955                 960

Thr Ile Gly Thr Gly Asp Gly Met Met Met Gln Thr Leu Ser Thr Met
                965                 970                 975

Lys Gly Ala Pro Lys Asn Thr Leu Lys Ile Phe Asp Gly Met Asn Ile
            980                 985                 990

Gly Leu Asn Asp Ile Thr Asp Ala Ser Arg Lys Ala Asn Glu Ala Val
        995                 1000                1005

Tyr Thr Ser Trp Gln Gly Asn Pro Ile Lys Asn Val Tyr Glu Ser Tyr
    1010                1015                1020

Ala Lys Phe Met Lys Asn Val Asp Phe Ser Lys Leu Ser Pro Glu Ala
1025                1030                1035                1040
```

Leu Glu Ala Ile Gly Lys Ser Ala Leu Glu Tyr Asp Gln Arg Glu Asn
1045 1050 1055

Ala Thr Val Asp Asp Ile Ala Asn Ala Ala Ser Leu Ile Glu Arg Asn
1060 1065 1070

Leu Arg Asn Ile Ala Leu Gly Val Asp Ile Arg His Lys Val Leu Asp
1075 1080 1085

Lys Val Asn Leu Ser Ile Asp Gln Met Ala Ala Val Gly Ala Pro Tyr
1090 1095 1100

Gln Asn Asn Gly Lys Ile Asp Leu Ser Asn Met Thr Pro Glu Gln Gln
1105 1110 1115 1120

Ala Asp Glu Leu Asn Lys Leu Phe Arg Glu Glu Leu Glu Ala Arg Lys
1125 1130 1135

Gln Lys Val Ala Lys Ala Arg
1140

<210> 7
<211> 3432
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 7
atggggggtt ctcatcatca tcatcatcat ggtatggcta gcatgactgg tggacagcaa 60
atgggtcggg atctgtacga cgatgacgat aaggatccga gctcgagatc tgaaagtaca 120
gttacagaag aattaaaaga aggtattgat gctgtttacc cttcattggt aggtactgct 180
gattctaaag cagagggtat taagaactat ttcaaattgt cctttacctt accagaagaa 240
cagaaatccc gtactgttgg ttcagaagca cctctaaaag atgtagccca agctctgtct 300
tctcgtgctc gttatgaact ctttactgag aaagaaactg ctaaccctgc ttttaatggg 360
gaagttatta agcgatacaa agaactcatg gaacatgggg aaggtattgc tgatattctt 420
cgctcccgtc tggctaagtt ccttaacact aaggatgttg gtaaacgttt tgctcaaggt 480
acagaagcca accgttgggt aggtggtaag ttacttaaca ttgttgagca ggatggggat 540
acctttaagt acaacgaaca attgctacag actgctgtat tagcaggtct tcaatggaga 600
cttactgcta ccagcaatac tgctatcaaa gatgcaaaag atgttgctgc tattactggt 660
attgaccaag ctctgctgcc agaaggttta gtagagcaat ttgatactgg tatgacactc 720
actgaagcag ttagttccct ggctcagaaa attgagtctt actggggatt atctcgtaat 780
ccaaatgctc cattgggcta taccaaaggc atccctacag caatggctgc tgaaattctg 840
gctgcatttg tagagtctac tgatgttgta gagaacatcg tggatatgtc agaaattgac 900
ccagataaca agaagactat tggtctgtac accattactg aactggattc cttcgaccca 960
attaatagct tccctactgc tattgaagaa gctgttttag tgaatcctac agagaagatg 1020
ttctttggtg atgacattcc tcctgtagct aatactcagc ttcgtaaccc tgctgttcgt 1080
aatactccag aacagaaggc tgcattgaaa gcagagcagg ctacagagtt ctatgtacac 1140
accccaatgg ttcaattcta tgagacgtta ggtaaagacc gtattctcga actgatgggt 1200
gctggtactc tgaataaaga gttacttaat gataaccatg ctaaatctct ggaaggtaag 1260
aaccgttcag tagaggactc ttacaaccaa ctgttctccg tcattgagca ggtaagagca 1320
cagagcgaag acatctctac tgtacctatt cactatgcat acaatatgac ccgtgttggt 1380
cgtatgcaga tgttaggtaa atacaatcct caatcagcca aactggttcg tgaggccatc 1440
ttacctacta aagctacttt ggatttatcg aaccagaaca atgaagactt ctctgcattc 1500
cagttaggtc tggctcaggc attggacatt aaagtccata ctatgactcg tgaggttatg 1560
tctgacgagt tgactaaatt actggaaggt aatctgaaac cagccattga tatgatggtt 1620
gagtttaata ccactggttc cttaccagaa aacgcagttg atgttctgaa tacagcatta 1680

ggagatagga agtcattcgt agcattgatg gctcttatgg agtattcccg ttacttagta 1740
gcagaggata aatctgcatt tgtaactcca ctgtatgtag aagcagatgg tgttactaat 1800
ggtccaatca atgccatgat gctaatgaca ggcggtctgt ttactcctga ctggattcgt 1860
aatattgcca aagggggctt gttcattggt tctccaaata agaccatgaa tgagcatcgc 1920
tctactgctg acaataatga tttatatcaa gcatccacta atgctttgat ggaatcgttg 1980
ggtaagttac gtagtaacta tgcctctaat atgcctattc agtctcagat agacagtctt 2040
ctttctctga tggatttgtt tttaccggat attaatcttg gtgagaatgg tgctttagaa 2100
cttaaacgtg gtattgctaa gaacccactg actattacca tcttcggttc tggtgctcgt 2160
ggtattgcag gtaagctggt tagttctgtt actgatgcca tctatgagcg tatgtctgat 2220
gtactgaaag ctcgtgctaa agacccaaat atctctgctg ctatggcaat gtttggtaag 2280
caagctgctt cagaagcaca tgctgaagaa cttcttgccc gtttcctgaa agatatggaa 2340
acactgactt ctactgttcc tgttaaacgt aaaggtgtac tggaactaca atccacaggt 2400
acaggagcca aaggaaaaat caatcctaag acctatacca ttaagggcga gcaactgaag 2460
gcacttcagg aaaatatgct gcacttcttt gtagaaccac tacgtaatgg tattactcag 2520
actgtaggtg aaagtctggt gtactctact gaacaattac agaaagctac tcagattcaa 2580
tctgtagtgc tggaagatat gttcaaacag cgagtacaag agaagctggc agagaaggct 2640
aaagacccaa catggaagaa aggtgatttc cttactcaga aagaactgaa tgatattcag 2700
gcttctctga ataacttagc ccctatgatt gagactggtt ctcagacttt ctacattgct 2760
ggttcagaaa atgcagaagt agcaaatcag gtattagcta ctaaccttga tgaccgtatg 2820
cgtgtaccaa tgagtatcta tgctccagca caggccggtg tagcaggtat tccatttatg 2880
actattggta ctggtgatgg catgatgatg caaactcttt ccactatgaa aggtgcacca 2940
aagaataccc tcaaaatctt tgatggtatg aacattggtt tgaatgacat cactgatgcc 3000
agtcgtaaag ctaatgaagc tgtttacact tcttggcagg gtaaccctat taagaatgtt 3060
tatgaatcat atgctaagtt catgaagaat gtagatttca gcaagctgtc ccctgaagca 3120
ttggaagcaa ttggtaaatc tgctctggaa tatgaccaac gtgagaatgc tactgtagat 3180
gatattgcta acgctgcatc tctgattgaa cgtaacttac gtaatattgc actgggtgta 3240
gatattcgtc ataaggtgct ggataaggta aatctgtcca ttgaccagat ggctgctgta 3300
ggtgctcctt atcagaacaa cggtaagatt gacctcagca atatgacccc tgaacaacag 3360
gctgatgaac tgaataaact tttccgtgaa gagttagaag cccgtaaaca aaaagtcgct 3420
aaggctaggt aa 3432

<210> 8
<211> 1143
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 8
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr
1 5 10 15

Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp
20 25 30

Pro Ser Ser Arg Ser Glu Ser Thr Val Thr Glu Glu Leu Lys Glu Gly
35 40 45

Ile Asp Ala Val Tyr Pro Ser Leu Val Gly Thr Ala Asp Ser Lys Ala
50 55 60

Glu Gly Ile Lys Asn Tyr Phe Lys Leu Ser Phe Thr Leu Pro Glu Glu
65 70 75 80

Gln Lys Ser Arg Thr Val Gly Ser Glu Ala Pro Leu Lys Asp Val Ala
85 90 95

Gln Ala Leu Ser Ser Arg Ala Arg Tyr Glu Leu Phe Thr Glu Lys Glu
100 105 110

Thr Ala Asn Pro Ala Phe Asn Gly Glu Val Ile Lys Arg Tyr Lys Glu
115 120 125

Leu Met Glu His Gly Glu Gly Ile Ala Asp Ile Leu Arg Ser Arg Leu
130 135 140

Ala Lys Phe Leu Asn Thr Lys Asp Val Gly Lys Arg Phe Ala Gln Gly
145 150 155 160

Thr Glu Ala Asn Arg Trp Val Gly Gly Lys Leu Leu Asn Ile Val Glu
165 170 175

Gln Asp Gly Asp Thr Phe Lys Tyr Asn Glu Gln Leu Leu Gln Thr Ala
180 185 190

Val Leu Ala Gly Leu Gln Trp Arg Leu Thr Ala Thr Ser Asn Thr Ala
195 200 205

Ile Lys Asp Ala Lys Asp Val Ala Ala Ile Thr Gly Ile Asp Gln Ala
210 215 220

Leu Leu Pro Glu Gly Leu Val Glu Gln Phe Asp Thr Gly Met Thr Leu
225 230 235 240

Thr Glu Ala Val Ser Ser Leu Ala Gln Lys Ile Glu Ser Tyr Trp Gly
245 250 255

Leu Ser Arg Asn Pro Asn Ala Pro Leu Gly Tyr Thr Lys Gly Ile Pro
260 265 270

Thr Ala Met Ala Ala Glu Ile Leu Ala Ala Phe Val Glu Ser Thr Asp
275 280 285

Val Val Glu Asn Ile Val Asp Met Ser Glu Ile Asp Pro Asp Asn Lys
290 295 300

Lys Thr Ile Gly Leu Tyr Thr Ile Thr Glu Leu Asp Ser Phe Asp Pro
305 310 315 320

Ile Asn Ser Phe Pro Thr Ala Ile Glu Glu Ala Val Leu Val Asn Pro
325 330 335

Thr Glu Lys Met Phe Phe Gly Asp Asp Ile Pro Pro Val Ala Asn Thr
340 345 350

Gln Leu Arg Asn Pro Ala Val Arg Asn Thr Pro Glu Gln Lys Ala Ala
355 360 365

Leu Lys Ala Glu Gln Ala Thr Glu Phe Tyr Val His Thr Pro Met Val
370 375 380

Gln Phe Tyr Glu Thr Leu Gly Lys Asp Arg Ile Leu Glu Leu Met Gly
385 390 395 400

Ala Gly Thr Leu Asn Lys Glu Leu Leu Asn Asp Asn His Ala Lys Ser
405 410 415

Leu Glu Gly Lys Asn Arg Ser Val Glu Asp Ser Tyr Asn Gln Leu Phe
420 425 430

Ser Val Ile Glu Gln Val Arg Ala Gln Ser Glu Asp Ile Ser Thr Val
435 440 445

Pro Ile His Tyr Ala Tyr Asn Met Thr Arg Val Gly Arg Met Gln Met
450 455 460

Leu Gly Lys Tyr Asn Pro Gln Ser Ala Lys Leu Val Arg Glu Ala Ile
465 470 475 480

Leu Pro Thr Lys Ala Thr Leu Asp Leu Ser Asn Gln Asn Asn Glu Asp
485 490 495

Phe Ser Ala Phe Gln Leu Gly Leu Ala Gln Ala Leu Asp Ile Lys Val
500 505 510

His Thr Met Thr Arg Glu Val Met Ser Asp Glu Leu Thr Lys Leu Leu
515 520 525

Glu Gly Asn Leu Lys Pro Ala Ile Asp Met Met Val Glu Phe Asn Thr
530 535 540

Thr Gly Ser Leu Pro Glu Asn Ala Val Asp Val Leu Asn Thr Ala Leu
545 550 555 560

Gly Asp Arg Lys Ser Phe Val Ala Leu Met Ala Leu Met Glu Tyr Ser
565 570 575

Arg Tyr Leu Val Ala Glu Asp Lys Ser Ala Phe Val Thr Pro Leu Tyr
580 585 590

Val Glu Ala Asp Gly Val Thr Asn Gly Pro Ile Asn Ala Met Met Leu
595 600 605

Met Thr Gly Gly Leu Phe Thr Pro Asp Trp Ile Arg Asn Ile Ala Lys
610 615 620

Gly Gly Leu Phe Ile Gly Ser Pro Asn Lys Thr Met Asn Glu His Arg
625 630 635 640

Ser Thr Ala Asp Asn Asn Asp Leu Tyr Gln Ala Ser Thr Asn Ala Leu
645 650 655

Met Glu Ser Leu Gly Lys Leu Arg Ser Asn Tyr Ala Ser Asn Met Pro
660 665 670

Ile Gln Ser Gln Ile Asp Ser Leu Leu Ser Leu Met Asp Leu Phe Leu
675 680 685

Pro Asp Ile Asn Leu Gly Glu Asn Gly Ala Leu Glu Leu Lys Arg Gly
690 695 700

Ile Ala Lys Asn Pro Leu Thr Ile Thr Ile Phe Gly Ser Gly Ala Arg
705 710 715 720

Gly Ile Ala Gly Lys Leu Val Ser Ser Val Thr Asp Ala Ile Tyr Glu
725 730 735

Arg Met Ser Asp Val Leu Lys Ala Arg Ala Lys Asp Pro Asn Ile Ser
740 745 750

Ala Ala Met Ala Met Phe Gly Lys Gln Ala Ala Ser Glu Ala His Ala
755 760 765

Glu Glu Leu Leu Ala Arg Phe Leu Lys Asp Met Glu Thr Leu Thr Ser
770 775 780

Thr Val Pro Val Lys Arg Lys Gly Val Leu Glu Leu Gln Ser Thr Gly
785 790 795 800

Thr Gly Ala Lys Gly Lys Ile Asn Pro Lys Thr Tyr Thr Ile Lys Gly
805 810 815

Glu Gln Leu Lys Ala Leu Gln Glu Asn Met Leu His Phe Phe Val Glu
820 825 830

Pro Leu Arg Asn Gly Ile Thr Gln Thr Val Gly Glu Ser Leu Val Tyr
835 840 845

Ser Thr Glu Gln Leu Gln Lys Ala Thr Gln Ile Gln Ser Val Val Leu
850 855 860

Glu Asp Met Phe Lys Gln Arg Val Gln Glu Lys Leu Ala Glu Lys Ala
865 870 875 880

Lys Asp Pro Thr Trp Lys Lys Gly Asp Phe Leu Thr Gln Lys Glu Leu
885 890 895

Asn Asp Ile Gln Ala Ser Leu Asn Asn Leu Ala Pro Met Ile Glu Thr
900 905 910

Gly Ser Gln Thr Phe Tyr Ile Ala Gly Ser Glu Asn Ala Glu Val Ala
915 920 925

Asn Gln Val Leu Ala Thr Asn Leu Asp Asp Arg Met Arg Val Pro Met
930 935 940

Ser Ile Tyr Ala Pro Ala Gln Ala Gly Val Ala Gly Ile Pro Phe Met
945 950 955 960

Thr Ile Gly Thr Gly Asp Gly Met Met Met Gln Thr Leu Ser Thr Met
965 970 975

Lys Gly Ala Pro Lys Asn Thr Leu Lys Ile Phe Asp Gly Met Asn Ile
980 985 990

Gly Leu Asn Asp Ile Thr Asp Ala Ser Arg Lys Ala Asn Glu Ala Val
995 1000 1005

Tyr Thr Ser Trp Gln Gly Asn Pro Ile Lys Asn Val Tyr Glu Ser Tyr
1010 1015 1020

Ala Lys Phe Met Lys Asn Val Asp Phe Ser Lys Leu Ser Pro Glu Ala
1025 1030 1035 1040

Leu Glu Ala Ile Gly Lys Ser Ala Leu Glu Tyr Asp Gln Arg Glu Asn
1045 1050 1055

Ala Thr Val Asp Asp Ile Ala Asn Ala Ala Ser Leu Ile Glu Arg Asn
1060 1065 1070

Leu Arg Asn Ile Ala Leu Gly Val Asp Ile Arg His Lys Val Leu Asp
1075 1080 1085

Lys Val Asn Leu Ser Ile Asp Gln Met Ala Ala Val Gly Ala Pro Tyr
1090 1095 1100

Gln Asn Asn Gly Lys Ile Asp Leu Ser Asn Met Thr Pro Glu Gln Gln
1105 1110 1115 1120

Ala Asp Glu Leu Asn Lys Leu Phe Arg Glu Glu Leu Glu Ala Arg Lys
1125 1130 1135

Gln Lys Val Ala Lys Ala Arg
1140

<210> 9
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 9
tcccagacaa aaggttaaga tttcatacag gattggatgc attacttcat ccaaaagaag 60
cggagcttc 69

<210> 10
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 10
tgggagagaa aaggttaaga tttgatagag gattggatgg attagttgat ggaaaagaag 60
cggagcttc 69

<210> 11
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 11
tccctgtctt ttggttttgt tttctttctg gtttggttgc ttttcttctt ccaaaagaag 60
cggagcttc 69

<210> 12
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 12
tcccacacaa aaccttaaca tttcatacac cattccatcc attacttcat ccaaaagaag 60
cggagcttc 69

<210> 13
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 13
acccagacaa aaggaaaaga aaacaaacag gaaaggaagc aaaacaacaa ccaaaagaag 60
cggagcttc 69

<210> 14
<211> 10617
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 14
atggggggtt ctcatcatca tcatcatcat ggtatggcta gcatgactgg tggacagcaa 60
atgggtcggg atctgtacga cgatgacgat aaggatccga gctcgagatc tatgtcagta 120
tttgatagac tggctgggtt cgcagacagc gtaaccaatg caaagcaagt tgacgtctct 180
actgcaaccg cccagaagaa agctgaacaa ggtgtcacta ctcctcttgt ttctcctgat 240
gctgcttatc aaatgcaagc tgcccgtact ggtaatgttg gggctaatgc atttgaacca 300
gggacagtgc aatcagattt catgaatctg accccaatgc aaatcatgaa taagtatggg 360
gttgagcaag gcttacaact tatcaatgct cgtgctgatg cagggaacca ggtattcaat 420
gattcagtta ctacaagaac tcctggggaa gaactggggg atattgctac tggtgttggc 480
cttggttttg ttaataccct tgggggcatt ggtgctcttg ggcaggctt actcaacgat 540
gatgcaggtg ctgttgttgc tcaacaattg agtaagttta atgatgctgt tcatgctacc 600
caaagccagg cattacaaga taaacgtaag ctctttgctg ctcgtaactt aatgaatgaa 660
gtagagagtg aacgtcagta tcaaacagat aagaaagaag gcactaatga catagtagct 720
tccttatcta aatttggacg tgattttgta ggttcaattg agaatgctgc tcaaactgac 780
tctattattt ctgatgggtt agcagaaggg gtaggttctc tattaggtgc tggtcctgta 840
ttaaggggtg catctttact gggtaaagca gttgttccag caaatactct tcgtagtgct 900
gcattggctg gtgctattga tgcaggtact ggtactcagt cactggctcg tattgcctct 960
actgtaggta gagctgcacc gggtatggtt ggtgttggtg caatggaagc tggtggtgca 1020
taccaacaaa ctgctgatga aattatgaag atgagtctta aagacttaga gaagtctcct 1080
gtttatcagc aacatattaa agatggtatg tcccctgaac aggctcgtcg tcagactgca 1140
tctgaaactg gtcttactgc tgctgctatt caattaccta ttgctgctgc aaccggtcct 1200
ctggtatccc gttttgagat ggctcctttc cgtgctggct ctttaggtgc tgtaggtatg 1260

aaccttgccc gtgaaacagt ggaagaaggt gttcagggtg ctacaggcca actggctcag 1320
aatattgcac agcaacaaaa cattgataag aaccaagacc tgcttaaagg tgtcggtaca 1380
caggctggtt taggtgctct ttatggcttt ggttctgctg gtgttgtaca ggctccggct 1440
ggtgctgctc gtttagcagg tgctgcaact gctcctgtat tgcgtaccac aatggctggt 1500
gttaaagctg ctggtagtgt agcaggtaag gttgtttctc ctattaagaa tactttagta 1560
gctcgtggtg aacgggttat gaagcagaat gaagaagcat ctcctgttgc tgatgactat 1620
gttgcacagg cagcacaaga agctatggct caagcaccag aagcagaagt tactattcgt 1680
gatgctgttg aagcaactga tgctactcca gaacagaaag ttgcagcaca ccagtatgtt 1740
tctgacttaa tgaatgctac tcgttttaat cctgaaaatt atcaggaagc accagagcat 1800
attcgtaatg ctgtagctgg ttctactgac caagtacagg ttattcagaa gttagcagac 1860
ttagttaaca cattagatga atctaatcct caagcactga tggaagctgc atcttatatg 1920
tatgatgctg tttcagagtt tgagcagttc attaaccgtg accctgctgc actggatagc 1980
attcctaaag attctccggc tattgagtta ctcaaccgtt atacgaatct gacagctaat 2040
attcagaaca caccaaaagt aattggtgca ctgaatgtta ttaatcgaat gattaatgaa 2100
tctgctcaga atggttcttt gaatgtgact gaagaatcca gtccacagga aatgcagaac 2160
gtagcattag ctgctgaagt agcccctgaa aagctcaatc cagagtctgt aaatgttgtt 2220
cttaaacatg ctgctgatgg tcgtattaaa ctgaataatc gccagattgc tgccctccag 2280
aatgctgctg caatcctgaa gggggcacgg gaatatgatg cagaagctgc ccgtcttgga 2340
ttacgtcctc aagacattgt gagtaaacag attaaaacgg atgagagcag aactcaggaa 2400
ggacaatact ctgcgttgca acatgcgaat aggattcggt ctgcgtataa ctctggtaat 2460
ttcgagttgg cctccgctta cctgaacgac tttatgcagt tcgcccagca catgcagaat 2520
aaggttggag cgttgaatga gcatcttgtt acggggaatg cggataagaa taagtctgtc 2580
cactaccaag ctcttactgc tgacagagaa tgggttcgta gccgtaccgg attgggggtc 2640
aatccctatg acactaagtc ggttaaattt gcccagcaag ttgctcttga agcgaaaacg 2700
gtagcggata ttgctaatgc cctcgcttcg gcttacccgg aactgaaggt cagtcatata 2760
aaagttactc cattggattc acgtcttaac gctcctgctg ctgaggtggt caaggcattc 2820
cgtcaaggca atcgagacgt tgcttcttct caaccgaaag ctgactccgt gaatcaggtt 2880
aaagaaactc ctgttacaaa acaggaacca gttacatcta ctgtacagac taagactcct 2940
gttagtgaat ctgttaaaac agaacctact actaaagagt ctagcccaca ggctataaaa 3000
gaacctgtga accagtctga aaaacaggat gttaacctta ctaatgagga caacatcaag 3060
caacctactg aatctgttaa agaaactgaa acttctacaa aagaaagtac agttacagaa 3120
gaattaaaag aaggtattga tgctgtttac ccttcattgg taggtactgc tgattctaaa 3180
gcagagggta ttaagaacta tttcaaattg tcctttacct taccagaaga acagaaatcc 3240
cgtactgttg gttcagaagc acctctaaaa gatgtagccc aagctctgtc ttctcgtgct 3300
cgttatgaac tctttactga gaaagaaact gctaaccctg cttttaatgg ggaagttatt 3360
aagcgataca aagaactcat ggaacatggg gaaggtattg ctgatattct tcgctcccgt 3420
ctggctaagt tccttaacac taaggatgtt ggtaaacgtt ttgctcaagg tacagaagcc 3480
aaccgttggg taggtggtaa gttacttaac attgttgagc aggatgggga tacctttaag 3540
tacaacgaac aattgctaca gactgctgta ttagcaggtc ttcaatggag acttactgct 3600
accagcaata ctgctatcaa agatgcaaaa gatgttgctg ctattactgg tattgaccaa 3660
gctctgctgc cagaaggttt agtagagcaa tttgatactg gtatgacact cactgaagca 3720
gttagttccc tggctcagaa aattgagtct tactggggat tatctcgtaa tccaaatgct 3780
ccattgggct ataccaaagg catccctaca gcaatggctg ctgaaattct ggctgcattt 3840
gtagagtcta ctgatgttgt agagaacatc gtggatatgt cagaaattga cccagataac 3900
aagaagacta ttggtctgta caccattact gaactggatt ccttcgaccc aattaatagc 3960
ttccctactg ctattgaaga agctgtttta gtgaatccta cagagaagat gttctttggt 4020
gatgacattc ctcctgtagc taatactcag cttcgtaacc ctgctgttcg taatactcca 4080
gaacagaagg ctgcattgaa agcagagcag gctacagagt tctatgtaca caccccaatg 4140
gttcaattct atgagacgtt aggtaaagac cgtattctcg aactgatggg tgctggtact 4200
ctgaataaag agttacttaa tgataaccat gctaaatctc tggaaggtaa gaaccgttca 4260
gtagaggact cttacaacca actgttctcc gtcattgagc aggtaagagc acagagcgaa 4320
gacatctcta ctgtacctat tcactatgca tacaatatga cccgtgttgg tcgtatgcag 4380
atgttaggta aatacaatcc tcaatcagcc aaactggttc gtgaggccat cttacctact 4440
aaagctactt tggatttatc gaaccagaac aatgaagact tctctgcatt ccagttaggt 4500
ctggctcagg cattggacat taaagtccat actatgactc gtgaggttat gtctgacgag 4560
ttgactaaat tactggaagg taatctgaaa ccagccattg atatgatggt tgagtttaat 4620
accactggtt ccttaccaga aaacgcagtt gatgttctga atacagcatt aggagatagg 4680
aagtcattcg tagcattgat ggctcttatg gagtattccc gttacttagt agcagaggat 4740
aaatctgcat ttgtaactcc actgtatgta gaagcagatg gtgttactaa tggtccaatc 4800
aatgccatga tgctaatgac aggcggtctg tttactcctg actggattcg taatattgcc 4860

aaagggggct tgttcattgg ttctccaaat aagaccatga atgagcatcg ctctactgct 4920
gacaataatg atttatatca agcatccact aatgctttga tggaatcgtt gggtaagtta 4980
cgtagtaact atgcctctaa tatgcctatt cagtctcaga tagacagtct tctttctctg 5040
atggatttgt ttttaccgga tattaatctt ggtgagaatg gtgctttaga acttaaacgt 5100
ggtattgcta agaacccact gactattacc atctatggtt ctggtgctcg tggtattgca 5160
ggtaagctgg ttagttctgt tactgatgcc atctatgagc gtatgtctga tgtactgaaa 5220
gctcgtgcta aagacccaaa tatctctgct gctatggcaa tgtttggtaa gcaagctgct 5280
tcagaagcac atgctgaaga acttcttgcc cgtttcctga aagatatgga aacactgact 5340
tctactgttc ctgttaaacg taaaggtgta ctggaactac aatccacagg tacaggagcc 5400
aaaggaaaaa tcaatcctaa gacctatacc attaagggcg agcaactgaa ggcacttcag 5460
gaaaatatgc tgcacttctt tgtagaacca ctacgtaatg gtattactca gactgtaggt 5520
gaaagtctgg tgtactctac tgaacaatta cagaaagcta ctcagattca atctgtagtg 5580
ctggaagata tgttcaaaca gcgagtacaa gagaagctgg cagagaaggc taaagaccca 5640
acatggaaga aaggtgattt ccttactcag aaagaactga atgatattca ggcttctctg 5700
aataacttag cccctatgat tgagactggt tctcagactt tctacattgc tggttcagaa 5760
aatgcagaag tagcaaatca ggtattagct actaaccttg atgaccgtat gcgtgtacca 5820
atgagtatct atgctccagc acaggccggt gtagcaggta ttccatttat gactattggt 5880
actggtgatg gcatgatgat gcaaactctt tccactatga aaggtgcacc aaagaatacc 5940
ctcaaaatct ttgatggtat gaacattggt ttgaatgaca tcactgatgc cagtcgtaaa 6000
gctaatgaag ctgtttacac ttcttggcag ggtaacccta ttaagaatgt ttatgaatca 6060
tatgctaagt tcatgaagaa tgtagatttc agcaagctgt cccctgaagc attggaagca 6120
attggtaaat ctgctctgga atatgaccaa cgtgagaatg ctactgtaga tgatattgct 6180
aacgctgcat ctctgattga acgtaactta cgtaatattg cactgggtgt agatattcgt 6240
cataaggtgc tggataaggt aaatctgtcc attgaccaga tggctgctgt aggtgctcct 6300
tatcagaaca acggtaagat tgacctcagc aatatgaccc ctgaacaaca ggctgatgaa 6360
ctgaataaac ttttccgtga agagttagaa gcccgtaaac aaaaagtcgc taaggctagg 6420
gctgaagtca aagaagaaac tgtttctgaa aaagaaccag tgaatccaga ctttggtatg 6480
gtaggccgtg agcataaggc atctggtgtt cgtatcctgt ctgctactgc tattcgtaat 6540
ctggctaaga ttagtaatct gccatctact caggcagcta ctcttgcgga gattcagaaa 6600
tcactggcag ctaaagacta taagattatc tacggtacac ctactcaggt tgcagagtat 6660
gctcgtcaga agaatgttac tgaattgact tctcaggaaa tggaagaagc tcaggcaggt 6720
aatatttatg gctggactaa cttcgatgat aagaccattt atctggttag cccatctatg 6780
gaaaccctca ttcatgaact ggttcatgcc tctaccttcg aggaagttta ttccttctat 6840
cagggtaatg aagtaagccc tacttctaag caggctattg agaaccttga aggtctgatg 6900
gaacagttcc gttctctgga tatttccaaa gattctccag aaatgagaga agcatatgct 6960
gatgctattg caactatcga aggtcatttg agtaatggat ttgttgaccc agctatctct 7020
aaagctgctg ctcttaatga gtttatggct tgggggttag ctaaccgtgc tcttgctgct 7080
aaacagaaga gaacatcttc actggttcaa atggtgaaag atgtttatca ggctattaag 7140
aaattgattt ggggacgtaa acaagctcct gcattgggag aagatatgtt ctccaatctg 7200
ctgtttaact ctgcaattct gatgcgtagc caacctacaa ctcaggcagt agctaaagat 7260
ggcacactgt tccatagcaa agcatatggt aataatgaac gtctgtctca gttgaaccag 7320
actttcgata aactggtaac tgattacctt cgtactgacc cagttacaga agtagaacgt 7380
cgtggcaatg tggctaatgc attaatgagt gctactcgac tggttcgtga tgttcagtct 7440
catggcttca atatgactgc tcaggaacag tctgtattcc agatggttac tgctgcatta 7500
gcaactgaag ctgcgattga cccacatgct atggctcgtg ctcaggaact ttatacccat 7560
gtaatgaaac accttacggt agagcatttc atggctgacc ctgatagtac taaccctgct 7620
gaccgttact atgctcaaca gaaatatgac accatctctg gtgctaatct ggttgaagta 7680
gatgccaaag gtagaaccag tctgttacct acattcctgg gtctggctat ggttaatgaa 7740
gaactacgtt caatcattaa agaaatgcct gtacctaaag cagataagaa attagggaat 7800
gatatagata ctctgcttac caatgcaggt actcaggtaa tggaatctct gaaccgtcgt 7860
atggctggtg accagaaagc tactaatgtt caggacagta ttgatgcttt gtcagaaaca 7920
atcatggctg ctgctttgaa acgagagtcc ttctatgatg ctgtagcaac ccctaccggt 7980
aacttcattg accgtgctaa tcagtacgta acggatagca ttgaacggtt atctgaaact 8040
gttattgaga aggcagataa ggtaattgct aacccttcta atatagctgc taaaggtgtt 8100
gctcatctgg ctaaactgac tgctgctatt gcatctgaaa aacagggtga aatagtggct 8160
cagggtgtta tgactgctat gaaccagggt aaagtatggc aacctttcca tgacttagtt 8220
aatgacattg ttggccgtac taagactaat gccaatgtct atgacttaat caaattggtt 8280
aagagccaga tttctcaaga ccgtcagcaa ttccgtgagc atttacctac agtcattgct 8340
ggtaagttct ctcgtaaatt gactgatacc gaatggtctg caatgcatac tggtttaggt 8400
aaaacagatt tagctgttct acgtgaaact atgagcatgg ctgaaattag agatttactc 8460

tcttcatcca agaaagtgaa agatgaaatc tctactctgg aaaaagagat tcagaaccaa 8520
gcaggtagaa actggaatct ggttcagaag aaatctaagc aactggctca atacatgatt 8580
atgggggaag taggtaataa cctccttcgt aatgcccatg ctattagtcg tttgttaggt 8640
gaacgtatta ctaatggtcc tgtggcagat gtagctgcta ttgataagct cattactttg 8700
tactctctgg aattgatgaa taagtctgac cgtgaccttt tgtcagaatt ggctcaatca 8760
gaagtggaag gtatggagtt ctccattgct tatatggttg gtcaacgtac tgaagagatg 8820
cgtaaagcta aaggtgataa ccgtactctg ctgaatcact ttaaaggcta tatccctgta 8880
gagaaccagc aaggtgtgaa tttgattatt gctgacgata aagagtttgc taagttaaat 8940
agccaatcct ttactcgtat tggtacttat caggggagca ctggtttccg tactggttct 9000
aaaggttatt acttcagccc agtagctgcc cgtgcccctt actctcaggg tattcttcag 9060
aacgttcgta atactgctgg tggtgtggat attggtactg gctttacgtt aggcactatg 9120
gttgctgggc gtattactga caaaccaacc gtagagcgta ttaccaaagc tctggctaaa 9180
ggtgagcgtg ggcgtgaacc actgatgcca atttataaca gcaaaggtca ggtagttgct 9240
tatgaacaat ccgttgaccc taatatgttg aagcacctaa accaagacaa tcactttgct 9300
aagatggttg gtgtatggcg tggtcgtcag gtggaagagg ctaaagcaca acgttttaat 9360
gacattctca ttgagcaatt acatgctatg tatgagaaag acattaaaga ctccagtgct 9420
aataaatctc aatatgtaaa cctgttaggt aaaattgatg acccagtact ggctgatgcg 9480
attaacctga tgaacattga gactcgtcat aaggccgaag aactcttcgg taaagatgag 9540
ttatgggttc gtagggatat gctgaatgat gcacttggct atcgtgctgc atctattggt 9600
gatgtgtgga ccggtaactc tcgttggtca cctagcaccc ttgatactgt taagaagatg 9660
ttcctcggtg cattcggtaa taaggcatat catgtagtaa tgaatgctga aaataccatt 9720
cagaacttag tgaaggacgc taagacagta attgttgtta aatctgttgt agtaccggca 9780
gttaacttcc ttgctaacat ctaccagatg attggacgtg gtgttcctgt taaagatatt 9840
gctgtgaaca ttcctcgtaa gacgtcagag attaatcagt atattaaatc tcgtttacgt 9900
cagattgatg cggaagcaga gctacgtgct gctgaaggta accctaatct ggttcgtaaa 9960
cttaaaactg agattcaatc tattactgat agtcatcgtc gtatgagtat ctggcctttg 10020
attgaagcag gtgagttctc ttctattgct gatgctggta ttagtcgtga tgacctgtta 10080
gtagctgaag gtaagattca tgagtacatg gaaaaacttg ctaataaact tccagaaaaa 10140
gtacgtaatg ctggccgtta cgctcttatt gctaaggaca ctgctctgtt ccagggtatc 10200
cagaaaacag tagagtattc agactttatt gctaaagcca tcatctatga tgatttagtg 10260
aaacgtaaga aaaaatcttc ttctgaagca ttaggtcagg taactgaaga gtttattaac 10320
tatgacagat tgcctggtcg tttccgtggc tatatggaaa gtatgggtct gatgtggttc 10380
tacaacttta aaattcgttc cattaaagtt gctatgagca tgattagaaa caacccagta 10440
cattctctga ttgctacagt agtacctgct cctaccatgt ttggtaacgt aggtctacca 10500
attcaggaca acatgctaac catgctggct gaaggaagac tggattactc attaggcttc 10560
ggacaaggat taagagcacc taccctcaat ccttggttca accttactca ctaataa 10617

<210> 15
<211> 3537
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Peptide

<400> 15
Met Gly Gly Ser His His His His His His Gly Met Ala Ser Met Thr
1 5 10 15

Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp
20 25 30

Pro Ser Ser Arg Ser Met Ser Val Phe Asp Arg Leu Ala Gly Phe Ala
35 40 45

Asp Ser Val Thr Asn Ala Lys Gln Val Asp Val Ser Thr Ala Thr Ala
50 55 60

Gln Lys Lys Ala Glu Gln Gly Val Thr Thr Pro Leu Val Ser Pro Asp
65 70 75 80

Ala Ala Tyr Gln Met Gln Ala Ala Arg Thr Gly Asn Val Gly Ala Asn
85 90 95

Ala Phe Glu Pro Gly Thr Val Gln Ser Asp Phe Met Asn Leu Thr Pro
100 105 110

Met Gln Ile Met Asn Lys Tyr Gly Val Glu Gln Gly Leu Gln Leu Ile
115 120 125

Asn Ala Arg Ala Asp Ala Gly Asn Gln Val Phe Asn Asp Ser Val Thr
130 135 140

Thr Arg Thr Pro Gly Glu Glu Leu Gly Asp Ile Ala Thr Gly Val Gly
145 150 155 160

Leu Gly Phe Val Asn Thr Leu Gly Gly Ile Gly Ala Leu Gly Ala Gly
165 170 175

Leu Leu Asn Asp Asp Ala Gly Ala Val Val Ala Gln Gln Leu Ser Lys
180 185 190

Phe Asn Asp Ala Val His Ala Thr Gln Ser Gln Ala Leu Gln Asp Lys
195 200 205

Arg Lys Leu Phe Ala Ala Arg Asn Leu Met Asn Glu Val Glu Ser Glu
210 215 220

Arg Gln Tyr Gln Thr Asp Lys Lys Glu Gly Thr Asn Asp Ile Val Ala
225 230 235 240

Ser Leu Ser Lys Phe Gly Arg Asp Phe Val Gly Ser Ile Glu Asn Ala
245 250 255

Ala Gln Thr Asp Ser Ile Ile Ser Asp Gly Leu Ala Glu Gly Val Gly
260 265 270

Ser Leu Leu Gly Ala Gly Pro Val Leu Arg Gly Ala Ser Leu Leu Gly
275 280 285

Lys Ala Val Val Pro Ala Asn Thr Leu Arg Ser Ala Ala Leu Ala Gly
290 295 300

Ala Ile Asp Ala Gly Thr Gly Thr Gln Ser Leu Ala Arg Ile Ala Ser
305 310 315 320

Thr Val Gly Arg Ala Ala Pro Gly Met Val Gly Val Gly Ala Met Glu
325 330 335

Ala Gly Gly Ala Tyr Gln Gln Thr Ala Asp Glu Ile Met Lys Met Ser
340 345 350

Leu Lys Asp Leu Glu Lys Ser Pro Val Tyr Gln Gln His Ile Lys Asp
355 360 365

Gly Met Ser Pro Glu Gln Ala Arg Arg Gln Thr Ala Ser Glu Thr Gly
370 375 380

Leu Thr Ala Ala Ala Ile Gln Leu Pro Ile Ala Ala Ala Thr Gly Pro
385 390 395 400

Leu Val Ser Arg Phe Glu Met Ala Pro Phe Arg Ala Gly Ser Leu Gly
405 410 415

Ala Val Gly Met Asn Leu Ala Arg Glu Thr Val Glu Glu Gly Val Gln
420 425 430

Gly Ala Thr Gly Gln Leu Ala Gln Asn Ile Ala Gln Gln Gln Asn Ile
435 440 445

Asp Lys Asn Gln Asp Leu Leu Lys Gly Val Gly Thr Gln Ala Gly Leu
450 455 460

Gly Ala Leu Tyr Gly Phe Gly Ser Ala Gly Val Val Gln Ala Pro Ala
465 470 475 480

Gly Ala Ala Arg Leu Ala Gly Ala Ala Thr Ala Pro Val Leu Arg Thr
485 490 495

Thr Met Ala Gly Val Lys Ala Ala Gly Ser Val Ala Gly Lys Val Val
500 505 510

Ser Pro Ile Lys Asn Thr Leu Val Ala Arg Gly Glu Arg Val Met Lys
515 520 525

Gln Asn Glu Glu Ala Ser Pro Val Ala Asp Asp Tyr Val Ala Gln Ala
530 535 540

Ala Gln Glu Ala Met Ala Gln Ala Pro Glu Ala Glu Val Thr Ile Arg
545 550 555 560

Asp Ala Val Glu Ala Thr Asp Ala Thr Pro Glu Gln Lys Val Ala Ala
565 570 575

His Gln Tyr Val Ser Asp Leu Met Asn Ala Thr Arg Phe Asn Pro Glu
580 585 590

Asn Tyr Gln Glu Ala Pro Glu His Ile Arg Asn Ala Val Ala Gly Ser
595 600 605

Thr Asp Gln Val Gln Val Ile Gln Lys Leu Ala Asp Leu Val Asn Thr
610 615 620

Leu Asp Glu Ser Asn Pro Gln Ala Leu Met Glu Ala Ala Ser Tyr Met
625 630 635 640

Tyr Asp Ala Val Ser Glu Phe Glu Gln Phe Ile Asn Arg Asp Pro Ala
645 650 655

Ala Leu Asp Ser Ile Pro Lys Asp Ser Pro Ala Ile Glu Leu Leu Asn
660 665 670

Arg Tyr Thr Asn Leu Thr Ala Asn Ile Gln Asn Thr Pro Lys Val Ile
675 680 685

Gly Ala Leu Asn Val Ile Asn Arg Met Ile Asn Glu Ser Ala Gln Asn
690 695 700

Gly Ser Leu Asn Val Thr Glu Glu Ser Ser Pro Gln Glu Met Gln Asn
705 710 715 720

Val Ala Leu Ala Ala Glu Val Ala Pro Glu Lys Leu Asn Pro Glu Ser
725 730 735

Val Asn Val Val Leu Lys His Ala Ala Asp Gly Arg Ile Lys Leu Asn
740 745 750

Asn Arg Gln Ile Ala Ala Leu Gln Asn Ala Ala Ala Ile Leu Lys Gly
755 760 765

Ala Arg Glu Tyr Asp Ala Glu Ala Ala Arg Leu Gly Leu Arg Pro Gln
770 775 780

Asp Ile Val Ser Lys Gln Ile Lys Thr Asp Glu Ser Arg Thr Gln Glu
785 790 795 800

Gly Gln Tyr Ser Ala Leu Gln His Ala Asn Arg Ile Arg Ser Ala Tyr
805 810 815

Asn Ser Gly Asn Phe Glu Leu Ala Ser Ala Tyr Leu Asn Asp Phe Met
820 825 830

Gln Phe Ala Gln His Met Gln Asn Lys Val Gly Ala Leu Asn Glu His
835 840 845

Leu Val Thr Gly Asn Ala Asp Lys Asn Lys Ser Val His Tyr Gln Ala
850 855 860

Leu Thr Ala Asp Arg Glu Trp Val Arg Ser Arg Thr Gly Leu Gly Val
865 870 875 880

Asn Pro Tyr Asp Thr Lys Ser Val Lys Phe Ala Gln Gln Val Ala Leu
885 890 895

Glu Ala Lys Thr Val Ala Asp Ile Ala Asn Ala Leu Ala Ser Ala Tyr
900 905 910

Pro Glu Leu Lys Val Ser His Ile Lys Val Thr Pro Leu Asp Ser Arg
915 920 925

Leu Asn Ala Pro Ala Ala Glu Val Val Lys Ala Phe Arg Gln Gly Asn
930 935 940

Arg Asp Val Ala Ser Ser Gln Pro Lys Ala Asp Ser Val Asn Gln Val
945 950 955 960

Lys Glu Thr Pro Val Thr Lys Gln Glu Pro Val Thr Ser Thr Val Gln
965 970 975

Thr Lys Thr Pro Val Ser Glu Ser Val Lys Thr Glu Pro Thr Thr Lys
980 985 990

Glu Ser Ser Pro Gln Ala Ile Lys Glu Pro Val Asn Gln Ser Glu Lys
995 1000 1005

Gln Asp Val Asn Leu Thr Asn Glu Asp Asn Ile Lys Gln Pro Thr Glu
1010 1015 1020

Ser Val Lys Glu Thr Glu Thr Ser Thr Lys Glu Ser Thr Val Thr Glu
1025 1030 1035 1040

Glu Leu Lys Glu Gly Ile Asp Ala Val Tyr Pro Ser Leu Val Gly Thr
1045 1050 1055

Ala Asp Ser Lys Ala Glu Gly Ile Lys Asn Tyr Phe Lys Leu Ser Phe
1060 1065 1070

Thr Leu Pro Glu Glu Gln Lys Ser Arg Thr Val Gly Ser Glu Ala Pro
1075 1080 1085

Leu Lys Asp Val Ala Gln Ala Leu Ser Ser Arg Ala Arg Tyr Glu Leu
1090 1095 1100

Phe Thr Glu Lys Glu Thr Ala Asn Pro Ala Phe Asn Gly Glu Val Ile
1105 1110 1115 1120

Lys Arg Tyr Lys Glu Leu Met Glu His Gly Glu Gly Ile Ala Asp Ile
1125 1130 1135

Leu Arg Ser Arg Leu Ala Lys Phe Leu Asn Thr Lys Asp Val Gly Lys
1140 1145 1150

Arg Phe Ala Gln Gly Thr Glu Ala Asn Arg Trp Val Gly Gly Lys Leu
1155 1160 1165

Leu Asn Ile Val Glu Gln Asp Gly Asp Thr Phe Lys Tyr Asn Glu Gln
1170 1175 1180

Leu Leu Gln Thr Ala Val Leu Ala Gly Leu Gln Trp Arg Leu Thr Ala
1185 1190 1195 1200

Thr Ser Asn Thr Ala Ile Lys Asp Ala Lys Asp Val Ala Ala Ile Thr
1205 1210 1215

Gly Ile Asp Gln Ala Leu Leu Pro Glu Gly Leu Val Glu Gln Phe Asp
1220 1225 1230

Thr Gly Met Thr Leu Thr Glu Ala Val Ser Ser Leu Ala Gln Lys Ile
1235 1240 1245

Glu Ser Tyr Trp Gly Leu Ser Arg Asn Pro Asn Ala Pro Leu Gly Tyr
1250 1255 1260

Thr Lys Gly Ile Pro Thr Ala Met Ala Ala Glu Ile Leu Ala Ala Phe
1265 1270 1275 1280

Val Glu Ser Thr Asp Val Val Glu Asn Ile Val Asp Met Ser Glu Ile
1285 1290 1295

Asp Pro Asp Asn Lys Lys Thr Ile Gly Leu Tyr Thr Ile Thr Glu Leu
1300 1305 1310

Asp Ser Phe Asp Pro Ile Asn Ser Phe Pro Thr Ala Ile Glu Glu Ala
1315 1320 1325

Val Leu Val Asn Pro Thr Glu Lys Met Phe Phe Gly Asp Asp Ile Pro
1330 1335 1340

Pro Val Ala Asn Thr Gln Leu Arg Asn Pro Ala Val Arg Asn Thr Pro
1345 1350 1355 1360

Glu Gln Lys Ala Ala Leu Lys Ala Glu Gln Ala Thr Glu Phe Tyr Val
1365 1370 1375

His Thr Pro Met Val Gln Phe Tyr Glu Thr Leu Gly Lys Asp Arg Ile
1380 1385 1390

Leu Glu Leu Met Gly Ala Gly Thr Leu Asn Lys Glu Leu Leu Asn Asp
1395 1400 1405

Asn His Ala Lys Ser Leu Glu Gly Lys Asn Arg Ser Val Glu Asp Ser
1410 1415 1420

Tyr Asn Gln Leu Phe Ser Val Ile Glu Gln Val Arg Ala Gln Ser Glu
1425 1430 1435 1440

Asp Ile Ser Thr Val Pro Ile His Tyr Ala Tyr Asn Met Thr Arg Val
1445 1450 1455

Gly Arg Met Gln Met Leu Gly Lys Tyr Asn Pro Gln Ser Ala Lys Leu
1460 1465 1470

Val Arg Glu Ala Ile Leu Pro Thr Lys Ala Thr Leu Asp Leu Ser Asn
1475 1480 1485

Gln Asn Asn Glu Asp Phe Ser Ala Phe Gln Leu Gly Leu Ala Gln Ala
1490 1495 1500

Leu Asp Ile Lys Val His Thr Met Thr Arg Glu Val Met Ser Asp Glu
1505 1510 1515 1520

Leu Thr Lys Leu Leu Glu Gly Asn Leu Lys Pro Ala Ile Asp Met Met
1525 1530 1535

Val Glu Phe Asn Thr Thr Gly Ser Leu Pro Glu Asn Ala Val Asp Val
1540 1545 1550

Leu Asn Thr Ala Leu Gly Asp Arg Lys Ser Phe Val Ala Leu Met Ala
1555 1560 1565

Leu Met Glu Tyr Ser Arg Tyr Leu Val Ala Glu Asp Lys Ser Ala Phe
1570 1575 1580

Val Thr Pro Leu Tyr Val Glu Ala Asp Gly Val Thr Asn Gly Pro Ile
1585 1590 1595 1600

Asn Ala Met Met Leu Met Thr Gly Gly Leu Phe Thr Pro Asp Trp Ile
1605 1610 1615

Arg Asn Ile Ala Lys Gly Gly Leu Phe Ile Gly Ser Pro Asn Lys Thr
1620 1625 1630

Met Asn Glu His Arg Ser Thr Ala Asp Asn Asn Asp Leu Tyr Gln Ala
1635 1640 1645

Ser Thr Asn Ala Leu Met Glu Ser Leu Gly Lys Leu Arg Ser Asn Tyr
1650 1655 1660

Ala Ser Asn Met Pro Ile Gln Ser Gln Ile Asp Ser Leu Leu Ser Leu
1665 1670 1675 1680

Met Asp Leu Phe Leu Pro Asp Ile Asn Leu Gly Glu Asn Gly Ala Leu
1685 1690 1695

Glu Leu Lys Arg Gly Ile Ala Lys Asn Pro Leu Thr Ile Thr Ile Tyr
1700 1705 1710

Gly Ser Gly Ala Arg Gly Ile Ala Gly Lys Leu Val Ser Ser Val Thr
1715 1720 1725

Asp Ala Ile Tyr Glu Arg Met Ser Asp Val Leu Lys Ala Arg Ala Lys
1730 1735 1740

Asp Pro Asn Ile Ser Ala Ala Met Ala Met Phe Gly Lys Gln Ala Ala
1745 1750 1755 1760

Ser Glu Ala His Ala Glu Glu Leu Leu Ala Arg Phe Leu Lys Asp Met
1765 1770 1775

Glu Thr Leu Thr Ser Thr Val Pro Val Lys Arg Lys Gly Val Leu Glu
1780 1785 1790

Leu Gln Ser Thr Gly Thr Gly Ala Lys Gly Lys Ile Asn Pro Lys Thr
1795 1800 1805

Tyr Thr Ile Lys Gly Glu Gln Leu Lys Ala Leu Gln Glu Asn Met Leu
1810 1815 1820

His Phe Phe Val Glu Pro Leu Arg Asn Gly Ile Thr Gln Thr Val Gly
1825 1830 1835 1840

Glu Ser Leu Val Tyr Ser Thr Glu Gln Leu Gln Lys Ala Thr Gln Ile
1845 1850 1855

Gln Ser Val Val Leu Glu Asp Met Phe Lys Gln Arg Val Gln Glu Lys
1860 1865 1870

Leu Ala Glu Lys Ala Lys Asp Pro Thr Trp Lys Lys Gly Asp Phe Leu
1875 1880 1885

Thr Gln Lys Glu Leu Asn Asp Ile Gln Ala Ser Leu Asn Asn Leu Ala
1890 1895 1900

Pro Met Ile Glu Thr Gly Ser Gln Thr Phe Tyr Ile Ala Gly Ser Glu
1905 1910 1915 1920

Asn Ala Glu Val Ala Asn Gln Val Leu Ala Thr Asn Leu Asp Asp Arg
1925 1930 1935

Met Arg Val Pro Met Ser Ile Tyr Ala Pro Ala Gln Ala Gly Val Ala
1940 1945 1950

Gly Ile Pro Phe Met Thr Ile Gly Thr Gly Asp Gly Met Met Met Gln
1955 1960 1965

Thr Leu Ser Thr Met Lys Gly Ala Pro Lys Asn Thr Leu Lys Ile Phe
1970 1975 1980

Asp Gly Met Asn Ile Gly Leu Asn Asp Ile Thr Asp Ala Ser Arg Lys
1985 1990 1995 2000

Ala Asn Glu Ala Val Tyr Thr Ser Trp Gln Gly Asn Pro Ile Lys Asn
2005 2010 2015

Val Tyr Glu Ser Tyr Ala Lys Phe Met Lys Asn Val Asp Phe Ser Lys
2020 2025 2030

Leu Ser Pro Glu Ala Leu Glu Ala Ile Gly Lys Ser Ala Leu Glu Tyr
2035 2040 2045

Asp Gln Arg Glu Asn Ala Thr Val Asp Asp Ile Ala Asn Ala Ala Ser
2050 2055 2060

Leu Ile Glu Arg Asn Leu Arg Asn Ile Ala Leu Gly Val Asp Ile Arg
2065 2070 2075 2080

His Lys Val Leu Asp Lys Val Asn Leu Ser Ile Asp Gln Met Ala Ala
2085 2090 2095

Val Gly Ala Pro Tyr Gln Asn Asn Gly Lys Ile Asp Leu Ser Asn Met
2100 2105 2110

Thr Pro Glu Gln Gln Ala Asp Glu Leu Asn Lys Leu Phe Arg Glu Glu
2115 2120 2125

Leu Glu Ala Arg Lys Gln Lys Val Ala Lys Ala Arg Ala Glu Val Lys
2130 2135 2140

Glu Glu Thr Val Ser Glu Lys Glu Pro Val Asn Pro Asp Phe Gly Met
2145 2150 2155 2160

Val Gly Arg Glu His Lys Ala Ser Gly Val Arg Ile Leu Ser Ala Thr
2165 2170 2175

Ala Ile Arg Asn Leu Ala Lys Ile Ser Asn Leu Pro Ser Thr Gln Ala
2180 2185 2190

Ala Thr Leu Ala Glu Ile Gln Lys Ser Leu Ala Ala Lys Asp Tyr Lys
2195 2200 2205

Ile Ile Tyr Gly Thr Pro Thr Gln Val Ala Glu Tyr Ala Arg Gln Lys
2210 2215 2220

Asn Val Thr Glu Leu Thr Ser Gln Glu Met Glu Glu Ala Gln Ala Gly
2225 2230 2235 2240

Asn Ile Tyr Gly Trp Thr Asn Phe Asp Asp Lys Thr Ile Tyr Leu Val
2245 2250 2255

Ser Pro Ser Met Glu Thr Leu Ile His Glu Leu Val His Ala Ser Thr
2260 2265 2270

Phe Glu Glu Val Tyr Ser Phe Tyr Gln Gly Asn Glu Val Ser Pro Thr
2275 2280 2285

Ser Lys Gln Ala Ile Glu Asn Leu Glu Gly Leu Met Glu Gln Phe Arg
2290 2295 2300

Ser Leu Asp Ile Ser Lys Asp Ser Pro Glu Met Arg Glu Ala Tyr Ala
2305 2310 2315 2320

Asp Ala Ile Ala Thr Ile Glu Gly His Leu Ser Asn Gly Phe Val Asp
2325 2330 2335

Pro Ala Ile Ser Lys Ala Ala Ala Leu Asn Glu Phe Met Ala Trp Gly
2340 2345 2350

Leu Ala Asn Arg Ala Leu Ala Ala Lys Gln Lys Arg Thr Ser Ser Leu
2355 2360 2365

Val Gln Met Val Lys Asp Val Tyr Gln Ala Ile Lys Lys Leu Ile Trp
2370 2375 2380

Gly Arg Lys Gln Ala Pro Ala Leu Gly Glu Asp Met Phe Ser Asn Leu
2385 2390 2395 2400

Leu Phe Asn Ser Ala Ile Leu Met Arg Ser Gln Pro Thr Thr Gln Ala
2405 2410 2415

Val Ala Lys Asp Gly Thr Leu Phe His Ser Lys Ala Tyr Gly Asn Asn
2420 2425 2430

Glu Arg Leu Ser Gln Leu Asn Gln Thr Phe Asp Lys Leu Val Thr Asp
2435 2440 2445

Tyr Leu Arg Thr Asp Pro Val Thr Glu Val Glu Arg Arg Gly Asn Val
2450 2455 2460

Ala Asn Ala Leu Met Ser Ala Thr Arg Leu Val Arg Asp Val Gln Ser
2465 2470 2475 2480

His Gly Phe Asn Met Thr Ala Gln Glu Gln Ser Val Phe Gln Met Val
2485 2490 2495

Thr Ala Ala Leu Ala Thr Glu Ala Ala Ile Asp Pro His Ala Met Ala
2500 2505 2510

Arg Ala Gln Glu Leu Tyr Thr His Val Met Lys His Leu Thr Val Glu
2515 2520 2525

His Phe Met Ala Asp Pro Asp Ser Thr Asn Pro Ala Asp Arg Tyr Tyr
2530 2535 2540

Ala Gln Gln Lys Tyr Asp Thr Ile Ser Gly Ala Asn Leu Val Glu Val
2545 2550 2555 2560

Asp Ala Lys Gly Arg Thr Ser Leu Leu Pro Thr Phe Leu Gly Leu Ala
2565 2570 2575

Met Val Asn Glu Glu Leu Arg Ser Ile Ile Lys Glu Met Pro Val Pro
2580 2585 2590

Lys Ala Asp Lys Lys Leu Gly Asn Asp Ile Asp Thr Leu Leu Thr Asn
2595 2600 2605

Ala Gly Thr Gln Val Met Glu Ser Leu Asn Arg Arg Met Ala Gly Asp
2610 2615 2620

Gln Lys Ala Thr Asn Val Gln Asp Ser Ile Asp Ala Leu Ser Glu Thr
2625 2630 2635 2640

Ile Met Ala Ala Ala Leu Lys Arg Glu Ser Phe Tyr Asp Ala Val Ala
2645 2650 2655

Thr Pro Thr Gly Asn Phe Ile Asp Arg Ala Asn Gln Tyr Val Thr Asp
2660 2665 2670

Ser Ile Glu Arg Leu Ser Glu Thr Val Ile Glu Lys Ala Asp Lys Val
2675 2680 2685

Ile Ala Asn Pro Ser Asn Ile Ala Ala Lys Gly Val Ala His Leu Ala
2690 2695 2700

Lys Leu Thr Ala Ala Ile Ala Ser Glu Lys Gln Gly Glu Ile Val Ala
2705 2710 2715 2720

Gln Gly Val Met Thr Ala Met Asn Gln Gly Lys Val Trp Gln Pro Phe
2725 2730 2735

His Asp Leu Val Asn Asp Ile Val Gly Arg Thr Lys Thr Asn Ala Asn
2740 2745 2750

Val Tyr Asp Leu Ile Lys Leu Val Lys Ser Gln Ile Ser Gln Asp Arg
2755 2760 2765

Gln Gln Phe Arg Glu His Leu Pro Thr Val Ile Ala Gly Lys Phe Ser
2770 2775 2780

Arg Lys Leu Thr Asp Thr Glu Trp Ser Ala Met His Thr Gly Leu Gly
2785 2790 2795 2800

Lys Thr Asp Leu Ala Val Leu Arg Glu Thr Met Ser Met Ala Glu Ile
2805 2810 2815

Arg Asp Leu Leu Ser Ser Ser Lys Lys Val Lys Asp Glu Ile Ser Thr
2820 2825 2830

Leu Glu Lys Glu Ile Gln Asn Gln Ala Gly Arg Asn Trp Asn Leu Val
2835 2840 2845

Gln Lys Lys Ser Lys Gln Leu Ala Gln Tyr Met Ile Met Gly Glu Val
2850 2855 2860

Gly Asn Asn Leu Leu Arg Asn Ala His Ala Ile Ser Arg Leu Leu Gly
2865 2870 2875 2880

Glu Arg Ile Thr Asn Gly Pro Val Ala Asp Val Ala Ala Ile Asp Lys
2885 2890 2895

Leu Ile Thr Leu Tyr Ser Leu Glu Leu Met Asn Lys Ser Asp Arg Asp
2900 2905 2910

Leu Leu Ser Glu Leu Ala Gln Ser Glu Val Glu Gly Met Glu Phe Ser
2915 2920 2925

Ile Ala Tyr Met Val Gly Gln Arg Thr Glu Glu Met Arg Lys Ala Lys
2930 2935 2940

Gly Asp Asn Arg Thr Leu Leu Asn His Phe Lys Gly Tyr Ile Pro Val
2945 2950 2955 2960

Glu Asn Gln Gln Gly Val Asn Leu Ile Ile Ala Asp Asp Lys Glu Phe
2965 2970 2975

Ala Lys Leu Asn Ser Gln Ser Phe Thr Arg Ile Gly Thr Tyr Gln Gly
2980 2985 2990

Ser Thr Gly Phe Arg Thr Gly Ser Lys Gly Tyr Tyr Phe Ser Pro Val
2995 3000 3005

Ala Ala Arg Ala Pro Tyr Ser Gln Gly Ile Leu Gln Asn Val Arg Asn
3010 3015 3020

Thr Ala Gly Gly Val Asp Ile Gly Thr Gly Phe Thr Leu Gly Thr Met
3025 3030 3035 3040

Val Ala Gly Arg Ile Thr Asp Lys Pro Thr Val Glu Arg Ile Thr Lys
3045 3050 3055

Ala Leu Ala Lys Gly Glu Arg Gly Arg Glu Pro Leu Met Pro Ile Tyr
3060 3065 3070

Asn Ser Lys Gly Gln Val Val Ala Tyr Glu Gln Ser Val Asp Pro Asn
3075 3080 3085

Met Leu Lys His Leu Asn Gln Asp Asn His Phe Ala Lys Met Val Gly
3090 3095 3100

Val Trp Arg Gly Arg Gln Val Glu Glu Ala Lys Ala Gln Arg Phe Asn
3105 3110 3115 3120

Asp Ile Leu Ile Glu Gln Leu His Ala Met Tyr Glu Lys Asp Ile Lys
3125 3130 3135

Asp Ser Ser Ala Asn Lys Ser Gln Tyr Val Asn Leu Leu Gly Lys Ile
3140 3145 3150

Asp Asp Pro Val Leu Ala Asp Ala Ile Asn Leu Met Asn Ile Glu Thr
3155 3160 3165

Arg His Lys Ala Glu Glu Leu Phe Gly Lys Asp Glu Leu Trp Val Arg
3170 3175 3180

Arg Asp Met Leu Asn Asp Ala Leu Gly Tyr Arg Ala Ala Ser Ile Gly
3185 3190 3195 3200

Asp Val Trp Thr Gly Asn Ser Arg Trp Ser Pro Ser Thr Leu Asp Thr
3205 3210 3215

Val Lys Lys Met Phe Leu Gly Ala Phe Gly Asn Lys Ala Tyr His Val
3220 3225 3230

Val Met Asn Ala Glu Asn Thr Ile Gln Asn Leu Val Lys Asp Ala Lys
3235 3240 3245

Thr Val Ile Val Val Lys Ser Val Val Val Pro Ala Val Asn Phe Leu
3250 3255 3260

Ala Asn Ile Tyr Gln Met Ile Gly Arg Gly Val Pro Val Lys Asp Ile
3265 3270 3275 3280

Ala Val Asn Ile Pro Arg Lys Thr Ser Glu Ile Asn Gln Tyr Ile Lys
3285 3290 3295

Ser Arg Leu Arg Gln Ile Asp Ala Glu Ala Glu Leu Arg Ala Ala Glu
3300 3305 3310

Gly Asn Pro Asn Leu Val Arg Lys Leu Lys Thr Glu Ile Gln Ser Ile
3315 3320 3325

Thr Asp Ser His Arg Arg Met Ser Ile Trp Pro Leu Ile Glu Ala Gly
3330 3335 3340

Glu Phe Ser Ser Ile Ala Asp Ala Gly Ile Ser Arg Asp Asp Leu Leu
3345 3350 3355 3360

Val Ala Glu Gly Lys Ile His Glu Tyr Met Glu Lys Leu Ala Asn Lys
3365 3370 3375

Leu Pro Glu Lys Val Arg Asn Ala Gly Arg Tyr Ala Leu Ile Ala Lys
3380 3385 3390

Asp Thr Ala Leu Phe Gln Gly Ile Gln Lys Thr Val Glu Tyr Ser Asp
3395 3400 3405

Phe Ile Ala Lys Ala Ile Ile Tyr Asp Asp Leu Val Lys Arg Lys Lys
3410 3415 3420

Lys Ser Ser Ser Glu Ala Leu Gly Gln Val Thr Glu Glu Phe Ile Asn
3425 3430 3435 3440

Tyr Asp Arg Leu Pro Gly Arg Phe Arg Gly Tyr Met Glu Ser Met Gly
3445 3450 3455

Leu Met Trp Phe Tyr Asn Phe Lys Ile Arg Ser Ile Lys Val Ala Met
3460 3465 3470

Ser Met Ile Arg Asn Asn Pro Val His Ser Leu Ile Ala Thr Val Val
3475 3480 3485

Pro Ala Pro Thr Met Phe Gly Asn Val Gly Leu Pro Ile Gln Asp Asn
3490 3495 3500

Met Leu Thr Met Leu Ala Glu Gly Arg Leu Asp Tyr Ser Leu Gly Phe
3505 3510 3515 3520

Gly Gln Gly Leu Arg Ala Pro Thr Leu Asn Pro Trp Phe Asn Leu Thr
3525 3530 3535

His

<210> 16
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 16
ggcattactt catccaaaag aagcggagct tc 32

<210> 17
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 17
ggccatccat tacttcatcc aaaagaagcg gagcttc 37

<210> 18
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 18
ggatccaaaa gaagcggagc ttc 23

<210> 19
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 19
ggcattactt catccaaaag aagctgagct tc 32

<210> 20
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 20
ggcattactt catccaaaag aagcggagc 29

<210> 21
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 21
ggaggctcct cggagtctcc tttt 24

<210> 22
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 22
ggactacctt cgggtagtcc ttttt 25

<210> 23
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 23
agaaggggc tactaagccc tcttcttatt ttt 33

<210> 24
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 24
aagctgctcc gcagctttt 19

<210> 25
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
Primer

<400> 25
aaggctatcc ctacgggggt agcctttatt ttttt 35

<210> 26
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 26
gccctccttg tgagggcttt tt 22

<210> 27
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 27
caacgaagcg ttgaatacct 20

<210> 28
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 28
ttcttcgagg cgaagaaaac ct 22

<210> 29
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 29
cgacgaggcg tcgaaaacca 20